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# DETERMINATION OF THE TOCOPHEROLS AND THE TOCOPHERYLQUINONES BY THE COLORIMETRIC OXIDATION-REDUCTION METHOD

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It is well known that the vitamin E activity of many materials diminishes under a variety of conditions and that the tocopherols are readily converted to the tocopherylquinones which are devoid of vitamin E activity (1). It seemed important, therefore, to be able to measure not only the tocopherol content, but also the amount of the vitamin destroyed by conversion to the quinone. This communication deals with the measurement of the tocopherol and the tocopherylquinone content of a number of substances as determined by the method reported for vitamin K (2).

The method for the determination of vitamin K has been described in some detail (3) and the influence of the tocopherylquinones upon the performance of the test has been discussed. More recently (4), it was shown that extrapolation of the readings to zero time gave the vitamin K content of the sample, and the final steady reading gave the sum of both vitamin K and the tocopherylquinones. The concentration of the latter is thus obtained by difference. Following a gold chloride oxidation of the tocopherols in the sample, the tocopherol concentration is measured by the same method.

## EXPERIMENTAL

The sample to be tested is dissolved in butanol and reduced over Raney's nickel catalyst in the presence of phenosafranine as the indicator. When the reduction is complete, the solution is pumped into an upper chamber which contains a standard solution of 2,6-dichloroindophenol in butanol. The blue color is reduced in part by the hydroquinone, and the diminution in color is a measure of the quinone originally present. Readings are taken in the Evelyn photometer equipped with Filter 660. Vitamin K hydroquinone reduces the indophenol instantaneously, whereas the reduction due to the tocopherylhydroquinones is completed only after 40 to 60 minutes. Serial readings are therefore taken. The reading at zero time gives the vitamin K content and the final steady reading gives the sum of the concentrations of vitamin K and the tocopherylquinones. A detailed description of the apparatus and the performance of the test has been given (3). It may be pointed out that reconstruction of the apparatus to smaller dimensions should increase the sensitivity of the method.



In order to apply this method to the determination of the tocopherols, it is only necessary to convert these compounds to the corresponding quinones. This is carried out as follows: Dissolve the sample, containing about 150 to 300  $\gamma$  of the tocopherols, in 10 cc. of reagent butanol and add 0.5 cc. of a 20 per cent aqueous solution of gold chloride<sup>1</sup> ( $\text{AuCl}_3 \cdot \text{HCl} \cdot 3\text{H}_2\text{O}$ ). Allow the solution to stand in the absence of light at room temperatures for 30 minutes. Add 10 cc. of water and 30 cc. of technical hexane (b.p. 65–70°) and shake the mixture in a separatory funnel. Draw off the aqueous layer and wash the organic layer with two successive 10 cc. portions of water followed by 10 cc. of carbonate buffer (pH 9.7) and complete the washing with an additional 10 cc. of water. Draw off the organic layer, centrifuge it to remove precipitated gold oxides, and dry the extract superficially with anhydrous sodium sulfate. Filter, and concentrate an aliquot of the extract to a small volume *in vacuo* under nitrogen. Add sufficient phenosafranine solution to give a final concentration of 0.5  $\gamma$  of the indicator per cc., adjust to a convenient volume with butanol-acetate solution, and analyze as previously described (2).

In a series of control experiments with solutions of pure  $\alpha$ -tocopherol the recovery was 102 per cent (average deviation,  $\pm 3$ ; maximum deviation,  $\pm 5$  per cent). A large excess of gold chloride (100 mg.) is used in this procedure, but when this excess was reduced to 10 mg. the recovery was 86 per cent ( $\pm 5$ , maximum deviation, 14 per cent), and the recoveries were somewhat lower with solutions of  $\beta$ -tocopherol. The use of this large excess of the gold chloride is particularly essential in work with oils or concentrates, since carotenoids and other substances are also oxidized by gold chlorides (1).

A number of oils known to possess vitamin E activity were examined. Comparatively large samples (0.1 to 1.0 cc.) were dissolved in 10 cc. of butanol (insoluble solids were removed) and analyzed directly for their tocopherylquinone content.<sup>2</sup> Smaller samples of the oils (0.05 to 0.3 cc.) dissolved in butanol were oxidized with gold chloride and analyzed for their tocopherol content.<sup>2</sup> Recovery experiments were also performed by adding pure  $\alpha$ -tocopherol to the oils in concentrations equal to those found in the samples. The data are shown in Table I.

The determination of the tocopherol and tocopherylquinone content of a given sample requires two analyses, but when the sample is small it is

<sup>1</sup> The gold can be recovered readily from the aqueous phases in subsequent operations.

<sup>2</sup> Extrapolation of the serial readings to zero time showed that the samples contained too little vitamin K to be measured; i.e., less than 50  $\gamma$  per cc. The samples undoubtedly contain considerably less vitamin K<sub>1</sub> than 50  $\gamma$  per cc. but it was not our object in this work to measure the vitamin K content of these samples.

not necessary to use two aliquots for these analyses. After the tocopherylquinone determination is performed, the quinone and unoxidized tocopherol in the final blue test solution can be recovered simply by adding 2 volumes of petroleum ether to the butanol solution and washing the indophenol from the organic phase with water containing a few drops of dilute sodium hydroxide. Two washes suffice to remove the indophenol. The petroleum ether may then be removed *in vacuo*, and the butanol solution adjusted to 10 cc. and oxidized with gold chloride. Determinations performed in this way have given results only about 5 per cent low.

A sample<sup>3</sup> of dog plasma was treated with an alkaline solution of formaldehyde and ethanol and extracted as described by Mayer and Sobotka (7).

TABLE I

*Tocopherol and Tocopherylquinone Concentrations (Expressed As  $\alpha$ -Tocopherol)*

Sample No.	Tocopherylquinone	Total tocopherol	Recovery	Data approximated from other sources*
	mg. per gm.	mg. per gm.	per cent	mg. per gm.
1. Crude wheat germ oil.....	0.17	4.2	99	
2. Medicinal wheat germ oil.....	0.14	3.2	92	4.5†
3. Crude corn oil.....	0.16	1.1	100	0.7-1.0 (5)
	mg. per cent			
4. Refined cottonseed oil.....	0.08	0.92	102	0.9 (6)
		mg. per cent		mg. per cent
5. Dog plasma .....	0.46	0.56		0.74 (7)
6. " " (petroleum ether extraction).....	0.38	0.57		
7. Whole human blood.....	0.32			
	0.30			
	0.31			

\* The figures in parentheses indicate bibliographic references.

† This value, given on the label of the sample, was obtained by bioassay.

The extract was washed with alkali and water, but the phosphatides were not removed, nor was the extract submitted to chromatographic adsorption to remove the carotenoids and vitamin A, since these substances do not interfere in the present procedure. Butanol was added to the ether extract, the ether was removed *in vacuo*, and aliquots of the butanol solution were analyzed with and without gold chloride oxidation. The total tocopherol found in the plasma is in reasonable agreement with the level

<sup>3</sup> A 40 cc. sample was taken. This permitted separate determinations of the tocopherols and the tocopherylquinones with samples each equivalent to 20 cc. of plasma. One such sample may be used for both determinations, as indicated earlier. No doubt samples considerably smaller than 20 cc. can be used if the apparatus is constructed to smaller dimensions.

reported by Mayer and Sobotka, but if the tocopherylquinone concentration is subtracted from the total tocopherol, since the bipyridine method cannot be used for quinones, the agreement is not good.<sup>4</sup> With extra precautions to avoid effects of light and oxidation, and with petroleum ether for the extraction instead of diethyl ether, the experiment was repeated with essentially the same results (Table I).

Apparently, heretofore, the tocopherylquinones have not been observed in blood. For this reason three samples (30 to 50 cc.) of whole human blood were refluxed for 5 minutes with 9 volumes of alcohol in the absence of light, and the precipitated proteins were extracted twice with 5 volumes of petroleum ether *in vacuo* under nitrogen. The combined extracts were concentrated and the residue was taken up in petroleum ether, and washed with a 1:1 solution of ethanol in water to remove extraneous colors. The concentrates were then transferred to butanol and analyzed without gold chloride oxidation. The results (Table I) appear to indicate that the tocopherylquinones exist in blood.

#### DISCUSSION

Although the test for the tocopherols described here is given in its simplest form, the substances measured must conform to a number of requirements of specificity. They must be oil-soluble, non-reducing substances which upon oxidation with gold chloride give rise to new substances capable of reversible reduction and oxidation, and the oxidation-reduction potential of these substances must be above that of phenosafranine and below that of 2,6-dichloroindophenol. These new substances must reduce the indophenol reagent at a characteristically slow rate. Carotenoids and vitamin A do not interfere. It would therefore appear that the method is quite specific for the tocopherols.

The specificity of the method can be increased by the use of the preliminary reductive treatment with Claisen's alkali.<sup>5</sup> This treatment has already been described and its use in the determination of the tocopherol and tocopherylquinone content of an alfalfa extract has been given (3). In this method, the concentrate, distributed between methanol and petroleum ether, is reduced over Raney's nickel. A strong solution of alcoholic potassium hydroxide is added, and the cryptophenolic tocopherols, which do not form potassium salts, are extracted with petroleum ether. The tocopherols are thus separated from acids, phenols, and other such closely related substances as the tocopherylhydroquinones, vitamin K<sub>1</sub> hydroquinone, etc.

<sup>4</sup> Dr. Sobotka kindly suggested that such variations may result from differences in the diets used.

<sup>5</sup> 50 gm. of potassium hydroxide in 25 cc. of water diluted to 100 cc. with methanol.

Parker and McFarlane (8) reported a method of separating extraneous colored materials from petroleum ether solutions of the tocopherols. This method, which involves extraction of the ethereal solution with 85 per cent sulfuric acid, removes vitamin K<sub>1</sub> and the tocopherylquinones without removing  $\alpha$ -tocopherol. With pure solutions of  $\beta$ -tocopherol, however, a yellow color is imparted to the acid phase, and low recoveries are obtained.

The preliminary reductive treatment with Claisen's alkali can be used to add to the specificity of the method for the determinations of the tocopherylquinones. After the tocopherols are separated by petroleum ether extraction of the alcoholic potassium hydroxide, the addition of water causes the hydrolysis of the potassium salts of the tocopherylhydroquinones and vitamin K hydroquinone. Only the polysubstituted hydroquinones which have oil-soluble groupings exhibit these cryptophenolic properties. These substances may thus be extracted with petroleum ether from the aqueous alcoholic potassium hydroxide which retains a wide variety of phenols, hydroquinones, etc. This added precaution was not necessary in the present study.

So far as we know, no attempts to measure both the tocopherol and tocopherylquinone content of a sample have been reported heretofore. No doubt the gold chloride titration method of Karrer and Keller (9) could be used. Following a preliminary reduction of the sample, both products would be measured, and a second titration without reduction would presumably give the tocopherol concentration. Since the Furter-Meyer method (10) can be used for both the quinones and the tocopherols, whereas the Emmerie-Engel method (11) is suitable only for the tocopherols, parallel determinations by these methods should give both the quinone and tocopherol concentrations by difference. The difficulties involved in these three methods—the lack of specificity, the use of chromatographic techniques to purify photolabile products, etc.—are too well known to require further discussion. It appears that the method described herein, possessing advantages of specificity and simplicity, makes the measurement of these products feasible.

A chemical method by means of which all three tocopherols are measured simultaneously cannot replace biological methods of assay. It is known that  $\alpha$ -tocopherol possesses greater vitamin E activity than the  $\beta$ - or  $\gamma$ -tocopherols (5) and that the relative proportions of these compounds vary in different materials (12). If a sample contains appreciable amounts of the  $\beta$ - and  $\gamma$ -tocopherols, chemical methods will give high values and only as the concentrations of these two tocopherols approach zero will the results of the chemical method approximate those of the animal assay procedure. Since the  $\beta$ - and  $\gamma$ -tocopherols possess equivalent biological activity, it is possible to group these substances for analysis, and if the

$\alpha$ - or the combined  $\beta$ - and  $\gamma$ -tocopherols could be determined separately, it would become possible to replace biological methods of assay by chemical methods. The  $\beta$ - and  $\gamma$ -tocopherols differ from the  $\alpha$ -tocopherols in that both possess unsubstituted positions ortho to the phenolic group. This suggests that nitrous acid or diazotized dinitroaniline might react with only the  $\beta$ - and  $\gamma$ -tocopherols present in a mixture of all three tocopherols.

Since this work is to be discontinued, the following observations are presented. Alcoholic solutions containing 20 mg. per cent of pure  $\beta$ - or  $\gamma$ -tocopherol, upon treatment with nitrous acid, gave evanescent purple colors which changed to a stable red color upon the addition of alkali. These reactions were not given by solutions of  $\alpha$ -tocopherol at concentrations as high as 100 mg. per cent. These reactions, believed to be due to oxidation of the more susceptible  $\beta$ - and  $\gamma$ -tocopherols to orthoquinones, may furnish a basis for the differentiation of the  $\alpha$ - and the  $\beta$ - and  $\gamma$ -tocopherols. Following this treatment the combined  $\beta$ - and  $\gamma$ -tocopherols can be measured colorimetrically or by oxidation-reduction methods.

#### SUMMARY

A colorimetric oxidation-reduction method for the quantitative determination of the tocopherylquinones and the tocopherols has been described. The same apparatus and materials required for the determination of vitamin K have been used. The specificity of the method has been discussed, and it has been shown that the tocopherylquinones as well as the tocopherols exist in significant amounts in a variety of materials. The difference in the biological activity between  $\alpha$ - and the  $\beta$ - and  $\gamma$ -tocopherols has been discussed and a method of differentiating the  $\beta$ - and  $\gamma$ -tocopherols from the  $\alpha$ -tocopherol has been suggested.

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# STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

## X. INDUCED VITAMIN C EXCRETION IN THE RAT AND ITS EFFECT ON THE HYPOPROTHROMBINEMIA CAUSED BY 3,3'-METHYLENEBIS-(4-HYDROXYCOUMARIN)\*

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The hemorrhagic and anticoagulant<sup>1</sup> agent 3,3'-methylenebis(4-hydroxycoumarin) (1, 2) induces a marked hypoprothrombinemia in rats especially when they are maintained on a basal ration low in certain dietary factors such as vitamin K (3). Experiments with rabbits suggested that *l*-ascorbic acid may also be a factor that diminishes the effectiveness of the anticoagulant. The administration of *l*-ascorbic acid to certain individuals prevented the hypoprothrombinemic action of single doses of the anticoagulant, although most susceptible rabbits developed the hypoprothrombinemia unless very large amounts of both *l*-ascorbic acid and 2-methyl-1,4-naphthoquinone were administered simultaneously (4). The present experiments deal with the effect of *l*-ascorbic acid on induced hypoprothrombinemia in rats, with particular emphasis on compounds which increase the excretion of vitamin C in this species.<sup>2</sup>

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<sup>1</sup> The term anticoagulant is used in the general sense that 3,3'-methylenebis(4-hydroxycoumarin) is an agent which, after action *in vivo*, impairs or prevents the coagulation of blood. It does not affect the clotting power when added to either blood or plasma *in vitro* (1).

<sup>2</sup> For purposes of this communication the term *l*-ascorbic acid refers only to the crystalline compound, while the term vitamin C refers to the substance in rat urine which reduces 2,6-dichlorophenol indophenol and cures scurvy in the guinea pig. *l*-Ascorbic acid has not been isolated from the urine of these rats (5, 6).

### Methods

The experimental procedure was essentially that described previously (3). Adult rats were fed a basal diet consisting either of an artificial ration (3) or of sweetened condensed milk.<sup>3</sup> After a fasting period of 12 hours, they were fed 2.5 mg. of 3,3'-methylenebis(4-hydroxycoumarin) contained in 2 gm. of ration. Blood samples were taken by heart puncture 24 hours later for the determination of the prothrombin time of 12.5 per cent plasma (7). The substances studied for modifying effects were either added to the basal ration for several days prior to the administration of the anticoagulant, or they were fed with the supplement. The excretion of vitamin C by rats on the various regimens was determined by the procedure developed and applied by others in comparable studies (5, 8).

### EXPERIMENTAL

*l-Ascorbic Acid and Prothrombin Time*—In the absence of anticoagulant the prothrombin time of 12.5 per cent rat plasma was 39 seconds (32 to 46). This average time was not influenced by feeding 25 mg. of *l*-ascorbic acid. When 2.5 mg. of anticoagulant were fed, the hypoprothrombinemia induced was likewise essentially the same with or without *l*-ascorbic acid; the prothrombin time of 12.5 per cent plasma from rats on the artificial ration ranged from 89 to 143 seconds, average 111, when the anticoagulant was given, as compared to an average of 105 seconds (range 64 to 153) when the supplement also contained 25 mg. of ascorbic acid. Similar results were obtained when 25 mg. of *l*-ascorbic acid were fed daily for 5 days before the ingestion of the anticoagulant, when the vitamin was injected intraperitoneally, or when the basal diet was condensed milk. Furthermore, *l*-ascorbic acid did not prolong the lives of rats receiving the anticoagulant daily (3). However, when the basal diet consisted of a grain ration (3), 25 mg. of *l*-ascorbic acid appeared to diminish the degree of hypoprothrombinemia induced. Prothrombin times averaged 46 seconds in the presence of *l*-ascorbic acid and 58 seconds when the anticoagulant was given alone. This probably indicates that the *l*-ascorbic acid had intensified the protective effect of the vitamin K present in the grain ration (4).

*Compounds Which Stimulate Excretion of Vitamin C*—Longenecker *et al.* (5, 6) in King's laboratory have observed that many compounds increase the excretion of vitamin C by rats maintained on a diet of condensed milk. The increased excretion was ascribed to a marked increase in the amounts of vitamin C synthesized. On the basis of this work it appeared possible that a greater local intracellular concentration of vitamin C might be achieved by the administration of potent compounds of the series used by

<sup>3</sup> Borden's Eagle brand.

King's coworkers than by the administration of *l*-ascorbic acid itself. Accordingly, representative compounds, carvone, chloretone, theobromine, aminopyrine, and paraldehyde, were fed to rats under the experimental conditions employed by Longenecker *et al.* (6). In addition single doses of 2.5 mg. of 3,3'-methylenebis(4-hydroxycoumarin) were administered, and the prothrombin time of 12.5 per cent plasma determined 24 hours later. The degree of hypoprothrombinemia induced was then compared with the amounts of vitamin C excreted. Although the various compounds did not alter the prothrombin level (or activity) of the plasma in the absence of the anticoagulant, the degree of protection against the induced hypoprothrombinemia was roughly proportional to the output of vitamin C in the urine (Table I); *i.e.*, compounds which stimulated the greatest excretion of vitamin counteracted the anticoagulant most effectively. Thus, 2.5 mg. of anticoagulant usually increased the prothrombin time by 62 seconds (from a normal value of 39 seconds to 101 seconds) but this increase was only 16 seconds (from the normal value of 39 seconds to 55 seconds) when 20 mg. of chloretone were ingested daily during the previous week. Carvone and aminopyrine, which increased the excretion of vitamin C appreciably, were also effective in counteracting the action of the anticoagulant, while paraldehyde showed both properties to a lesser degree (Table I). Usually, the amounts of vitamin excreted were the same as those previously reported (5, 6). However, when theobromine was given, most of the animals excreted an average of 2.0 mg. of vitamin C daily, and they were partially resistant to the action of the anticoagulant. Three individuals, on the contrary, showed no resistance to the anticoagulant, and they failed to excrete additional vitamin C.

Certain other observations also suggested a connection between vitamin C synthesis and resistance to the anticoagulant. Four of fifteen rats which had been fed carvone continued to excrete from 1.5 to 3.0 mg. of vitamin C per day for 2 weeks after carvone was no longer fed, and a corresponding resistance to single doses of the anticoagulant persisted: prothrombin time, 65 seconds after 1 week and 75 seconds after 2 weeks. Usually, the excretion of vitamin C decreased rapidly when the stimulating agent was removed from the diet. Attempts to replace the basal diet of condensed milk with an artificial ration (3) resulted in very irregular responses both in vitamin excretion when carvone was fed, and in the resistance to the anticoagulant.

Nevertheless, the parallelism between vitamin C excretion and resistance to induced hypoprothrombinemia was not always a rigid one. Thus, the greatest resistance to the anticoagulant was observed when 50 mg. of carvone were fed daily for 16 days, and under these conditions 7.0 mg. of vitamin C were excreted daily. In the presence of chloretone much larger



amounts of vitamin were excreted, although the resistance to the anti-coagulant was somewhat less; *i.e.*, the prothrombin times were greater (Table I).

Single doses of carvone or chloretone up to 150 mg. failed to counteract the hypoprothrombinemia induced by 3,3'-methylenebis(4-hydroxycoumarin), although as much as 4.5 mg. of vitamin C were excreted during the next 24 hours. Apparently, therefore, the "vitamin C stimulators" must be administered continuously before the effects of the anticoagulant

TABLE I

*Effect of Chloretone and of Biologically Related Compounds on Hypoprothrombinemia Induced by 2.5 Mg. of 3,3'-Methylenebis(4-hydroxycoumarin)*

Compound	Daily dose*	Ascorbic acid excreted daily†	Prothrombin time of 12.5 per cent plasma	No. of determinations
	mg.	mg.	sec.	
None		0.3	101 (79-132)‡	22
Chloretone	5	0.4	103 (101-105)	2
	10	10.6	77 (57- 97)	4
	20	25.5	55 (43- 68)	3
Carvone	2		119 (101-137)	2
	5	2.6	93 (66-112)	4
	10	2.0	78 (40-127)	6
	25	1.9	70 (47- 84)	3
	50	6.7	67 (50- 86)	6
	50	7.0	51 (47- 59)	3
Aminopyrine	20	6.2	60 (45- 76)	2
Paraldehyde	20	2.6	78 (75- 83)	3
Theobromine	20	0.3	97 (92-102)	3
	20	2.0	71 (54-129)	10

\* The compounds were fed for 7 days except in the last experiment for carvone, in which carvone was fed for 16 days.

† Average excretion on 7th day of feeding.

‡ In the absence of anticoagulant, the prothrombin time of 12.5 per cent rat plasma averages 39 seconds.

are counteracted, thus differentiating this group of compounds from substances of the vitamin K class, which are active in the rat in single doses (3).

*Survival of Rats Receiving Anticoagulant Daily*—Carvone and chloretone not only counteracted the hypoprothrombinemia induced by single doses of anticoagulant, but they also prolonged the lives of adult rats fed condensed milk plus 2.0 mg. of anticoagulant daily. The anticoagulant was given as a 1 per cent mixture in cooked corn-starch plus cottonseed oil. Carvone, chloretone solution, or *l*-ascorbic acid solution was stirred into the daily allotment of milk. Six rats on the anticoagulant alone died in 12 days

(range 7 to 17 days), and three receiving 40 mg. of *l*-ascorbic acid in addition died in 11 days (range 10 to 13 days). However, four of six rats receiving 10 mg. of carvone daily survived for 40 days, as did two of three receiving 20 mg. of chloretone daily. Individuals in the latter groups survived the effects of 2 mg. of anticoagulant daily for 75 days, when the experiment was discontinued. The survival of rats receiving carvone or chloretone was similar to that when 5 mg. of 2-methyl-1,4-naphthoquinone were fed in addition to the anticoagulant and condensed milk, although this amount of vitamin K furnished complete protection against the anticoagulant to rats on an artificial ration (3).

*3,3'*-Methylenebis(4-hydroxycoumarin) and Vitamin C Excretion—Rats on condensed milk excreted additional amounts of vitamin C in the urine when the anticoagulant was fed (Table II). As little as 1 mg. of anticoagulant resulted in a measurable increase in vitamin excretion, and this is also the minimum dose of anticoagulant detectable by an increase in prothrombin

TABLE II

*Urinary Excretion of Vitamin C by Rats Receiving 3,3'-Methylenebis(4-hydroxycoumarin) and Condensed Milk*<sup>1</sup>

Anticoagulant fed	No. of animals	Daily excretion of vitamin C
mg.		mg.
0.0	22	0.27 (0.12-0.46)
1.0	3	1.01 (0.80-1.40)
5.0	3	1.20 (1.13-1.24)
10.0	2	2.10 (2.04-2.15)
15.0	2	2.05 (1.51-2.58)
20.0	3	3.03 (1.10-4.15)

time of 12.5 per cent rat plasma (3). When 20 mg. of anticoagulant were fed, vitamin C excretion averaged 3.03 mg. during the subsequent 24 hours. There was no obvious connection between the volume of urine voided and the excretion of vitamin C, and the increased excretion was observed only on a diet of condensed milk. Increased excretion of vitamin C did not occur when the artificial basal ration was fed. However, 3,3'-methylenebis(4-hydroxycoumarin) differed from the many compounds of the carvone-chloretone series in that successive doses of the anticoagulant resulted in a decrease of the vitamin C excreted, whereas carvone, for example, can be administered daily for many weeks, with a continued high excretion of vitamin C (6). When 5 mg. of the anticoagulant were fed, most of the additional vitamin C excreted appeared in the urine from the 6th to the 9th hours, with decreasing amounts excreted thereafter. 18 hours after the ingestion of anticoagulant, the excretion of vitamin C was approximately equal to that before the anticoagulant was given.

This suggested that the anticoagulant was causing a temporary transfer of the vitamin from the tissues into the urine, rather than stimulating continued synthesis. Accordingly, the vitamin C content of various tissues from adult rats was determined, and compared with analyses from rats fed one or three doses of 25 mg. of anticoagulant each. The vitamin C of the blood was increased and fluctuations were noted in the vitamin content of lungs and adrenals (Table III), but the variations within groups were large, and no clear cut evidence was obtained of a depleting effect of the anticoagulant on the tissues.

TABLE III

*Effect of 3,3'-Methylenebis(4-hydroxycoumarin) on Vitamin C Content of Rat Tissues*

The results are given in mg. of vitamin C per gm. of tissue. Each value is an average of three determinations.

25 mg. doses of anticoagulant	None	1 dose	3 doses
Liver .....	0.20 (0.16-0.23)	0.26 (0.21-0.29)	0.20 (0.18-0.24)
Lung .....	0.21 (0.18-0.26)	0.19 (0.12-0.24)	0.16 (0.13-0.19)
Brain .....	0.31 (0.30-0.33)	0.36 (0.32-0.40)	0.34 (0.33-0.35)
Spleen .....	0.40 (0.36-0.43)	0.46 (0.38-0.58)	0.43 (0.40-0.45)
Small intestines .....	0.34 (0.29-0.38)	0.39 (0.34-0.45)	0.32 (0.27-0.37)
Adrenals .....	3.90 (3.75-4.06)	3.93 (3.62-4.40)	2.86 (2.08-3.40)
Blood .....	0.70 (0.50-0.89)	1.47 (1.09-1.85)	1.21 (1.09-1.35)

## DISCUSSION

In spite of the many functions ascribed to *l*-ascorbic acid, and the many clinical conditions which appear to respond favorably to its administration (9, 10), there have been only occasional and very fragmentary reports linking it to blood coagulation (11, 12). Nevertheless, the present results suggest that substances which stimulate the synthesis of vitamin C in the rat also affect the mechanism by which the hypoprothrombinemia induced by 3,3'-methylenebis(4-hydroxycoumarin) is counteracted. The explanation of this relationship, however, is at present obscure, since each of the two phenomena involved remains itself essentially unexplained. It would follow, however, that further information on the biological synthesis of either vitamin C or of prothrombin might aid in clarifying our conception of the other, even though there does not appear to be any obvious common factor in the two syntheses. Both have been postulated to occur in the liver, and hence the same liver proteins may be involved. In this connection Rose, Harris, and Chen (13) have found evidence of liver damage in rats given large doses of 3,3'-methylenebis(4-hydroxycoumarin). In fact, the liver of the rat appeared to be more susceptible to this type of lesion than any other tissue of any other species examined.

The clinical administration of large doses of *L*-ascorbic acid has been reported to hasten the healing of wounds (14, 15) and the cure of skin lesions due to bismuth or to arsenic poisoning (16). The addition of *L*-ascorbic acid to cultures of fibroblasts hastens the formation of collagen fibrils (17). Since each of these processes involves the synthesis of new protein, the postulated connection between vitamin C and prothrombin is not without parallel. Vitamin C is generally believed to function in the capillaries in the final solidification of intercellular cement substances, the essential components of which are present in severely scorbutic individuals. A somewhat analogous function might be ascribed to the vitamin in the biosynthesis of prothrombin, if it be assumed that prothrombin is formed in the body through a chain of reactions, one of which brings about the union of relatively large components. Thus the activation of prothrombin *per se* might involve either vitamin C or K or both.

#### SUMMARY

1. The administration of *L*-ascorbic acid failed to alter the hypoprothrombinemia induced in rats by the ingestion of 3,3'-methylenebis(4-hydroxycoumarin). It also failed to prolong the lives of rats receiving the anticoagulant plus condensed milk or an artificial diet daily.

2. Chloretone, carvone, and other substances which stimulate the excretion of vitamin C in the rat markedly lowered the degree of hypoprothrombinemia induced in rats by 3,3'-methylenebis(4-hydroxycoumarin). In general the capacity of a compound to promote the excretion of vitamin C paralleled its tendency to counteract the anticoagulant. Carvone and chloretone markedly prolonged the lives of rats ingesting the anticoagulant daily.

3. The anticoagulant alone caused a temporary increase in the excretion of vitamin C by rats maintained on condensed milk. The maximum effect was noted 6 to 9 hours after the ingestion of anticoagulant. Repeated doses, however, failed to maintain an increased excretion of the vitamin. The ingestion of anticoagulant produced an increase in the concentration of vitamin C in the blood but no significant changes in the content of other tissues.

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# THE QUANTITATIVE DETERMINATION OF LANTHIONINE

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On boiling wool for a short time with a dilute  $\text{Na}_2\text{CO}_3$  solution and then hydrolyzing the washed wool residue with  $\text{HCl}$ , Horn, Jones, and Ringel (1) isolated from the hydrolysate a crystalline thio ether amino acid which they named lanthionine. The structure of this compound they believed to be  $\beta$ -amino- $\beta$ -carboxyethyl sulfide,  $\text{COOH}-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$ , and this was confirmed by its synthesis by du Vigneaud and Brown (2). Subsequently Horn and Jones (3) isolated lanthionine from human hair, chicken feathers, and lactalbumin which had been treated with boiling 2 per cent  $\text{Na}_2\text{CO}_3$  and hydrolyzed with 20 per cent  $\text{HCl}$ . Then du Vigneaud, Brown, and Bonsnes (4) found that amorphous insulin similarly treated yielded lanthionine. Horn and Jones (3) were able to isolate lanthionine from wool when 0.1  $N$   $\text{NaOH}$  or 2 per cent  $\text{Na}_2\text{S}$  was used in place of the 2 per cent  $\text{Na}_2\text{CO}_3$  but when  $N$   $\text{NaOH}$  was used only cystine was found. In all of this work the lanthionine was isolated and no attempt was made to determine quantitatively the amount which was formed.

Lanthionine resembles methionine in that it is also a thio ether. Baernstein (5) has shown that  $\text{HI}$  will convert methionine into the thiolactone of homocysteine. By analogy lanthionine when boiled with  $\text{HI}$  should yield 1 mole of cysteine. If such were the case, it should be possible to determine quantitatively lanthionine alone or in the presence of cystine. Lanthionine does not react in the Sullivan (6) cystine or cysteine reactions; hence, if either of these were present, it could be determined first after  $\text{HCl}$  hydrolysis and then again following hydrolysis with  $\text{HI}$ . The amount of lanthionine present would be measured by the increase in cysteine found after the  $\text{HI}$  hydrolysis.

## EXPERIMENTAL

The lanthionine, prepared from wool by the procedure used by Horn, Jones, and Ringel (1), crystallized in the typical triangular plates. The conversion of lanthionine to cysteine was carried out by dissolving 25 mg. of the compound in 6.0 cc. of 57 per cent  $\text{HI}$  containing 1.0 per cent  $\text{KH}_2\text{PO}_2$  and heating the solution at  $135-140^\circ$  for 4 hours. During the entire period of heating a stream of nitrogen was bubbled through the solution. It is important that the 57 per cent  $\text{HI}$  solution employed be completely color-

less or else low values will result. Kassell and Brand (7) describe a method for the purification of the 57 per cent HI which can be employed or a portion of the HI solution can simply be heated with the addition of 1.0 per cent  $\text{KH}_2\text{PO}_2$  until it becomes colorless. Table I gives the results on the recovery of cysteine from lanthionine in three experiments. In each case the cysteine was determined in an aliquot of the hydrolysate by the Sullivan (8) method. With pure lanthionine the average recovery of the cysteine from the lanthionine is 98 per cent. Following these experiments several sets of mixtures of the three sulfur-containing amino acids, lanthionine, methionine, and cystine, were prepared in which the amount of

TABLE I

*Recovery of Lanthionine Alone and in Mixtures of Amino Acids*

Composition of Mixture A, lanthionine 50 mg., cystine 10 mg., methionine 10 mg.; Mixture B, lanthionine 10 mg., cystine 10 mg., methionine 10 mg.; Mixture C, lanthionine 10 mg., cystine 50 mg., methionine 50 mg.

Substance	Cysteine equivalent of lanthionine	Cysteine found less cystine from added cystine*	Per cent recovery
	mg.	mg.	
Lanthionine, 25 mg.....	14.54	14.68	101
"    25 " .....	14.54	14.25	98
"    25 " .....	14.54	13.99	96
Mixture A.....	29.0	28.3	98
"    " .....	29.0	28.2	98
"    " .....	29.0	27.9	96
"    B .....	5.8	5.62	97
"    " .....	5.8	5.58	96
"    " .....	5.8	5.58	96
"    " .....	5.8	5.63	97
"    C .....	5.8	5.8	100

\* The recovery of cystine following the HI hydrolysis is 98 per cent and this figure was used in correcting the amount of cysteine to be subtracted due to the cystine which had been added.

lanthionine was varied so that it was much higher than the amount of the other acids (Mixture A), just the same (Mixture B), or much less (Mixture C). The results are given in Table I. It will be seen that irrespective of the mixture employed neither cystine nor methionine interferes with the determination of lanthionine. The conversion of methionine to homocysteine by the HI procedure does not interfere with the Sullivan reaction for cysteine. The recovery of cystine following the HI treatment results in a 98 per cent recovery of cysteine and due allowance was always made for the amount of cystine which was in the mixture. The average recovery of lanthionine in all the mixtures was 97.3 per cent.

*Lanthionine Content of Alkali-Treated Wool and Lactalbumin*—The samples of alkali-treated wool and lactalbumin were prepared by boiling 3 gm. of each protein in 75 cc. of 2.0 per cent  $\text{Na}_2\text{CO}_3$  for  $\frac{1}{2}$  hour. The treated wool was washed ten times with 150 cc. portions of distilled water and, after being squeezed dry, was placed in a vacuum desiccator over  $\text{H}_2\text{SO}_4$ . The lactalbumin (prepared from lactalbumin Labco 7-HAAX) was precipitated from the sodium carbonate solution by the addition of acetic acid. The precipitated lactalbumin was washed and dried in a manner similar to that employed for the wool. Table II gives the data on the cystine and lanthionine content of the two proteins both before and after treatment with sodium carbonate. The cystine values for the untreated and the treated proteins were obtained after 6 hours hydrolysis with 20 per cent  $\text{HCl}$ . All values are corrected for moisture and ash.

As with the control experiments, the cysteine equivalent of the cystine present in the treated protein was subtracted from the total cysteine found

TABLE II

*Cystine and Lanthionine Content of Lactalbumin and Wool before and after Treatment with 2.0 Per Cent Sodium Carbonate*

Protein	Cystine	Lanthionine
	<i>per cent</i>	<i>per cent</i>
Wool .....	12.82	0.0
“ alkali-treated .....	2.41	5.62
Lactalbumin .....	3.71	0.0
“ alkali-treated .....	0.71	1.98

in the  $\text{HI}$  hydrolysate and the difference was considered to be due to lanthionine. In the alkali-treated wool the total sulfur, corrected for moisture and ash, was 1.93 per cent as compared with 3.52 per cent in the original wool. The sulfur content of the  $\text{HI}$  hydrolysate of the alkali-treated wool was 1.70 per cent. The cystine sulfur plus the lanthionine sulfur of the alkali-treated wool accounted for 78.2 per cent of the sulfur of the treated wool and 88.8 per cent of the sulfur in the hydrolysate of the treated wool. Evidently some of the sulfur present is lost during acid hydrolysis, possibly as  $\text{H}_2\text{S}$  from unremoved sodium sulfide.

The sulfur content of the  $\text{HCl}$  hydrolysate of the alkali-treated lactalbumin was 1.19 per cent as compared with 1.42 per cent in the original protein. The methionine sulfur in lactalbumin (Labco 7-HAAX) according to Kassell and Brand (7) is 0.62 per cent. When their figure is used for the methionine sulfur and ours for the cystine and the lanthionine, the total is 1.11 per cent sulfur or 93 per cent of the total sulfur of the hydrolyzed sample.



## SUMMARY

Lanthionine is converted to cysteine by boiling with HI. Lanthionine in mixtures containing both cystine and methionine can be estimated with a high degree of accuracy. Lanthionine formed by dilute alkali treatment of a protein, such as wool or lactalbumin, can be quantitatively estimated colorimetrically by first hydrolyzing the lanthionine-containing protein with HCl. The cystine present can be determined by the Sullivan procedure without interference from lanthionine. Then by hydrolysis of the protein with HI the total cysteine can be determined. The difference between the two hydrolysates gives the cysteine derived from lanthionine. Multiplying by the factor 1.72 gives the amount of lanthionine.

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# THE EFFECT OF CERTAIN ANALOGUES OF CHOLINE ON PEROSIS

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Recent studies of the biological activity of choline analogues have related to the prevention of fatty livers in rats and mice, to the prevention of hemorrhagic kidneys in rats, and to the conversion of homocysteine to methionine *in vivo*. The results have been summarized and discussed in recent articles and reviews (1-4). It has also been shown (5-9) that choline promotes growth and prevents perosis in chicks and turkeys. In the latter species, arsenocholine has an action similar to that of choline, but betaine is completely ineffective (6). The effect of compounds related to choline on growth and perosis in chicks has been studied further, and is reported in the present communication.

## EXPERIMENTAL

*Preparation of Compounds*—Ethyltrimethyl- $\beta$ -hydroxyethylammonium chloride (monoethylcholine) was prepared by treatment of trimethylaminoethanol with ethyl iodide followed by crystallization from acetone, solution in water, and treatment with silver chloride.

$C_6H_{15}ONCl$	Calculated.	Halogen	23.07
153.5	Found.	"	23.27, 23.38

Diethylmethyl- $\beta$ -hydroxyethylammonium chloride (diethylcholine) was prepared by treatment of diethylaminoethanol with methyl iodide and conversion of the product to chloride with silver chloride.

$C_7H_{15}ONCl$	Calculated.	Halogen	21.15
167.5	Found.	"	21.26, 21.25, 21.29

Triethyl- $\beta$ -hydroxyethylammonium chloride (triethylcholine) was similarly prepared except for the use of ethyl iodide rather than methyl iodide.

$C_8H_{19}ONCl$	Calculated.	Halogen	19.51
181.5	Found.	"	19.56, 19.65

Trimethylethylacetalammonium chloride was prepared by treatment of aminoethanacetal with methyl iodide and methyl sulfate, followed by treatment with silver chloride.

TABLE I

*Results Obtained with Chicks by Adding Choline and Its Analogues to Choline-Deficient Diet*

All weights are expressed in terms of the chlorides.

Series No.	Supplement to 100 gm. basal diet		Per cent incidence of perosis at			Gain in 28 days
			14 days	21 days	28 days	
		gm.				gm.
1	None		40	60	80	86
1	Choline	0.03	11	44	44	123
1	"	0.05	12	62	38	120
1	"	0.1	0	0	0	166
1	Betaine aldehyde	0.1	33	50	62	116
1	Diethylcholine	0.12	0	0	0	83
1	Triethylcholine	0.13	22	78	89	89
				24 days		
2	None			57	71	48
2	Choline	0.1		10	0	152
2	Arsenocholine	0.13		0	12	133
			15 days	21 days		
3	None		25	75	100	74
3	$\beta$ -Methylcholine	0.11	0	33	57	75
3	Monoethylcholine	0.11	0	0	0	121
3	Choline	0.1	0	0	11	142
			14 days			
4	None		19	45	89	77
4	$\beta$ -Methylcholine	0.17	0	12	14	69
4	Monoethylcholine	0.17	0	0	0	100
4	Choline	0.1	0	0	0	150
4	" + Diethylcholine	0.1 0.12	0 0	0 0	0 0	157
				22 days		
5	None		40	55	87	69
5	Betaine	0.3	30	75	75	105
5	Choline	0.1	0	0	0	149
			17 days	21 days		
6	None		11	38	50	52
6	Betaine	0.3	44	44	88	87
6	Choline	0.1	0	0	0	141

TABLE I—*Concluded*

Series No.	Supplement to 100 gm. basal diet		Per cent incidence of perosis at			Gain in
			13 days	19 days	28 days	28 days
		gm.				gm.
7	None			57	100	54
7	$\alpha, \alpha$ -Dimethylcholine	0.12		38	100	58
7	Choline	0.1		0	0	145
				21 days	25 days	In 25 days
8	None		20	100	100	69
8	Betaine aldehyde	0.15	60	89	89	99
8	Choline	0.1	0	0	0	124

$C_5H_{12}O_2NCl$ . Calculated. Halogen 16.75, N 6.62  
 211.5 Found. " 17.26, 17.21, N 6.53, 6.64

Trimethylethanalammmonium chloride (betaine aldehyde) was prepared by treatment of the ethanacetal with concentrated hydrochloric acid at room temperature for 12 hours, followed by removal of most of the hydrochloric acid by distillation under reduced pressure and neutralization of the remainder of the acid.

Trimethyl- $\beta$ -hydroxypropylammmonium chloride ( $\beta$ -methylcholine) was kindly supplied by Merck and Company, Inc., trimethyl- $\beta$ -hydroxyethylarsonium chloride (arsenocholine) by Hoffmann-La Roche, Inc., and trimethyl- $\beta$ -hydroxyisobutylammmonium chloride ( $\alpha, \alpha$ -dimethylcholine) by Dr. Vincent du Vigneaud.

*Biological Test*—Day-old chicks were placed in electrically heated battery brooders and were fed the experimental diets immediately. The basal diet consisted of glucose (cerelose) 53 parts, washed casein 18, gelatin 8 dried yeast (Anheuser-Busch, Strain G) 6, gum arabic U.S.P. 5, salt mixture<sup>1</sup> 5, crude soy bean oil 5, fish oil blend (3000 units of vitamin A, 400 of D per gm.) 0.3. The chicks were weighed and examined for perosis at frequent intervals as previously described (5, 6). The test period lasted 4 weeks, and from eight to ten chicks were used in each group. On the basal diet growth was slow, and symptoms of perosis were usually evident at 10 to 14 days of age. On the positive control diet, which consisted of the basal diet, 100 gm., plus choline chloride, 0.1 gm., growth was about twice as rapid as on the basal diet, and protection against perosis was usually complete. The substances to be tested were dissolved in 50 per cent ethanol and the solutions were incorporated in the diet. All choline analogues were fed as chlorides.

<sup>1</sup> Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, 42, 180 (1939).

### Results

The experimental data are summarized in Table I and the relative activities of the various compounds are summarized in Table II. Betaine when fed at a higher level, 1 gm. per 100 gm. of diet, was found to depress growth and to have no effect on perosis. The exhalations of the chicks which received arsenocholine had a pronounced odor of garlic.

TABLE II  
*Comparison of Choline with Certain of Its Analogues When Added to Choline-Deficient Diet and Fed to Chicks*

Compound	Activity in	
	Prevention of perosis	Promotion of growth
Choline.....	Excellent	Excellent
Arsenocholine.....	"	Good
Monoethylcholine.....	"	"
Diethylcholine.....	"	None
$\beta$ -Methylcholine.....	Moderate	"
Betaine.....	None	Slight
" aldehyde.....	"	"
Triethylcholine.....	"	None
$\alpha, \alpha$ -Dimethylcholine.....	"	"

### DISCUSSION

The relative effects of 54 compounds in transmethylation, in prevention of hemorrhagic kidneys and fatty livers, in prevention of perosis, and in promotion of growth of chicks have been summarized in tabular form and discussed by Moyer and du Vigneaud (4). The relative biological activities of certain choline analogues were also discussed by Welch and Landau (10). These authors drew attention to the fact that arsenocholine may substitute for choline in the biosynthesis of lecithin. Arsenocholine is apparently unable to convert homocystine to methionine *in vivo*; so that the promotion of growth and prevention of perosis by arsenocholine in chicks may not involve transmethylation.

The failure of diethylcholine to promote growth is presumably not due to a toxic effect on chicks, for no depression of growth was observed when this substance was fed in combination with choline (Table I).

Through the kindness of Dr. Sidney Colowick it has been found in the following experiments carried out by him that the liver of the chick possesses the ability to oxidize choline and its arsenic analogue.

The liver extracts were prepared from 4 gm. of liver obtained from a 6 week-old chick by grinding the tissue with sand and sodium phosphate

buffer (pH 7.4) to give a final concentration of either 0.04 or 0.06 M. In all cases 1 cc. of extract, representing 0.2 gm. of fresh liver, was used in standard Warburg vessels maintained at 37°; the final volume, after suitable additions, was 2 cc. Choline consumed 1.25 atoms of oxygen per mole in one experiment (5.8 hours) and 1.12 atoms in another (5 hours). With arsenocholine the initial rates were about one-third those with choline; a total of 0.51 atom of oxygen per mole was consumed in each experiment. During the experiments arsenocholine liberated a volatile gas with a garlic-like odor, and acid permanganate (0.002 M or 0.005 M) contained in a side arm of the Warburg vessel was decolorized, as previously found by Mann *et al.* (11) with rat liver. However, in contrast to their experiments with rat liver, semicarbazide reduced but did not prevent the development of the garlic-like odor from arsenocholine treated with chick liver extract. Treatment of arsenocholine with the chick liver extract under anaerobic conditions in Thunberg tubes also yielded the garlic-like odor. As in the aerobic experiments, less odor was produced in the presence of semicarbazide.

The oxygen consumption of chick liver extract was not appreciably increased in the presence of betaine or its arsenic analogue. A similar result was reported (11) for betaine in the case of rats. Odor was not produced when arsenobetaine was incubated with chick liver extract. However when arsenobetaine is administered orally to rats and chicks the exhalations and tissues have a typical garlic-like odor. The possibility that this may be due to the breakdown of arsenobetaine in the digestive tract has not been investigated. The available data indicate that the liver of the chick oxidizes choline and its arsenic analogue and that a secondary reaction occurs in the case of arsenocholine which results in the formation of a volatile arsenical compound, presumably trimethylarsine. The production of the volatile compound also occurred anaerobically. It was apparently not completely suppressed by semicarbazide, which may indicate a difference between the chick and the rat.

#### SUMMARY

1. A number of analogues of choline were fed to chicks as supplements to a basal diet that was deficient in choline. By this means the growth-promoting and antiperotic activities of the compounds were compared with those of choline.

2. Arsenocholine and monoethylcholine promoted growth to a marked extent, although not to as marked an extent as choline. Betaine and betaine aldehyde had a slight growth-promoting activity. The following compounds failed to promote growth: diethylcholine, triethylcholine,  $\beta$ -methylcholine, and  $\alpha,\alpha$ -dimethylcholine.

3. Arsenocholine, monoethylcholine, and diethylcholine were as effective as choline in preventing perosis.  $\beta$ -Methylcholine had a slight anti-perotic activity. The other compounds were ineffective in the prevention of perosis.

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# ELECTRON MICROGRAPHS OF PROTEIN MOLECULES

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## PLATES 1 AND 2

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The electron microscope is finding wide-spread use in the examination of preparations of bacteria, bacteriophages, and viruses, as well as in the determination of fine structures that exist in larger organisms and in various organic and inorganic materials (1-10). However, only a few electron micrographs of protein molecules have been made (11), despite the fact that the sizes of several protein molecules, as estimated by such indirect methods as sedimentation and diffusion, have been found to be larger than the present limit of resolution of the electron microscope (12-14). It appeared desirable, therefore, to examine by means of the electron microscope a few preparations of proteins of different molecular sizes in order to determine the degree of correlation between the molecular sizes and shapes estimated by indirect methods and those obtained by direct mensuration. Since the imaging of such small particles depends largely on contrast relations, studies of particles of silver (high density and therefore high contrast) mixed with tobacco mosaic virus were first made. These were followed by investigations of preparations of bushy stunt virus, edestin, *Busycon canaliculatum* hemocyanin, *Limulus polyphemus* hemocyanin, and *Viviparus malleatus* hemocyanin. Micrographs of these preparations showed particles having sizes in reasonable agreement with the molecular sizes estimated by indirect methods. Furthermore, it was possible to determine the approximate shapes of the molecules with some assurance. All of the pictures were taken with the RCA type B electron microscope without a limiting objective aperture, and with 60 kilovolt-electrons.

## EXPERIMENTAL

*Silver Nitrate and Tobacco Mosaic Virus*—A dilute solution of silver nitrate containing 0.01 mg. of ultracentrifugally purified tobacco mosaic virus per cc. was applied to a supporting collodion membrane and allowed to dry in the usual manner (9). Fig. 1 is a highly magnified reproduction of an electron micrograph of this preparation. The rods of tobacco mosaic

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virus 150 Å. wide and of low contrast are seen scattered over the field, together with numerous small particles, presumably of silver, formed during the preparation and evaporation of the mount. The fact that square images of silver particles can be recognized as such down to about 100 Å. in diameter and hexagons down to 150 Å. suggests (15) that the resolution is about 30 Å. in this picture. Particles of silver give high contrast images with bright halos as long as their apparent diameters are greater than about 100 Å. Dense spherical particles with diameters smaller than the limit of resolution appear to produce images with areas increasing only slightly with the diameter of the particle, but with densities increasing in proportion to the area of the particle. Thus, the smallest image of a silver particle in Fig. 1 which is about 45 Å. in diameter could be produced by a totally opaque silver particle only 15 Å. in diameter, for the opacity, proportional to the cross-sectional area of the particle ( $1/4\pi \times 15^2$  sq. Å.), would be spread over the area of the image ( $1/4\pi \times 45^2$  sq. Å.) to produce the 10 per cent contrast ( $15^2/45^2 = 0.11$ ) in the image necessary for the recognition of a small image.

Because of their lower density and the resulting lower contrast in the image, organic materials are much less favorable. Thus, the tobacco mosaic virus particles, about 150 Å. thick, appear in the image with only about twice the contrast of the smallest detectable silver particle, although they are much more easily recognized because the image occupies a larger area. Individual spherical particles of organic material might well need to have diameters as large as 60 to 75 Å. before they would be detectable in the present electron microscopes, not because the image would be too small, but because the contrast of the images of smaller particles would be too low. On the other hand, it might be expected that images covering large areas might require less than 10 per cent contrast to be detectable, so that thin films of organic material 30 Å. thick producing only 5 per cent contrast over a large area of the image could be detected.

*Bushy Stunt Virus*—Bushy stunt virus, purified by differential centrifugation, was mounted on a collodion membrane in 0.1 M phosphate buffer, the excess washed off in distilled water, and the remainder allowed to dry. A micrograph reproduced as Fig. 2 shows 113 particles having an average diameter of 26  $\mu$ . The diameters of 80 per cent of these do not differ measurably from the average figure. In several instances the particles are clumped together in groups of two, three, or more particles, but definite centers of density make it possible to resolve these into the constituent individual particles. The two patches of very dense areas and the two patches of very faint areas in the upper right of the micrograph were neglected in the calculations. The two light circular areas at the left center are due to holes in the collodion membrane. As a result of detailed

physicochemical studies on bushy stunt virus, it has been concluded that the molecules are homogeneous with respect to size, shape, and density, and that the spherical molecules are  $26\text{ m}\mu$  in diameter (16, 17). The results obtained by means of the electron microscope therefore confirm those obtained earlier by indirect methods.

*Edestin*—A saturated solution of a commercial preparation of edestin in dilute sodium chloride was mounted on a collodion membrane and washed forty times in distilled water. A typical micrograph obtained from this mount is shown in Fig. 3. It may be seen that large particles are almost completely absent and that most of the material appears to exist in the form of particles ranging up to about  $11\text{ m}\mu$  in diameter. This result is in accord with that obtained by von Ardenne (11) and with the molecular weight of about 300,000 calculated from sedimentation, diffusion, and specific volume data obtained in Svedberg's laboratory (13). However, on the basis of a dissymmetry factor of 1.2, which has been suggested for edestin, the particles would be rods about 24 by  $5\text{ m}\mu$  in size (14). Although the shapes of some of the particles shown in Fig. 3 may be regarded as evidence of departure from a spherical shape, it seems likely that a final decision regarding the probable asymmetry of the edestin molecule will require a more detailed study with the electron microscope.

*Busycon canaliculatum Hemocyanin*—The hemocyanin, kindly supplied by Dr. W. C. Boyd, was mounted at a concentration of  $10^{-6}$  gm. per cc. in water and the micrograph shown as Fig. 4 was obtained. The average diameter of 74 particles is about  $22\text{ m}\mu$  and 54 of these have diameters between 19 and  $24\text{ m}\mu$ . Eriksson-Quensel and Svedberg found preparations of *Busycon* hemocyanin to contain material having sedimentation constants of 13.5, 61.1, and 101.7 (12). The preparation used in the present study was examined in the analytical centrifuge by Dr. M. A. Lauffer and found to contain three distinct components. About 40 per cent of the dissolved material had a sedimentation constant of 18.9, about 20 per cent a sedimentation constant of 63.5, and about 40 per cent a sedimentation constant of 102.0. On the basis of spherical shapes, these components would have particle diameters of about 10, 19, and  $24\text{ m}\mu$ , respectively. Although six or seven of the particles shown in Fig. 4 have diameters of around  $10\text{ m}\mu$ , the proportion of such small particles is smaller than would be indicated by the centrifuge data. If a real difference exists, it is possible that it might be due to aggregation of the smaller particles during the process of drying the mounted preparation. Svedberg (13) lists a dissymmetry factor of 1.4 for the smallest component; hence, another possible explanation for the failure to picture many small particles might reside in the smallest particles being either too thin to give recognizable images if the molecules are oblate spheroids or too narrow if the molecules are prolate.

A dissymmetry factor of 1.2 is given for the  $S_{20} = 101.7$  component (13). On the basis of the usual calculation for a prolate ellipsoid of revolution, this would yield molecular dimensions of about 15 by 66  $m\mu$  (14). Since the particles shown in Fig. 4 do not have these dimensions, it appears more likely that the asymmetry is of the plate rather than of the rod-like type. The fact that the molecules appear much less dense than the spherical particles of bushy stunt virus shown in Fig. 2 might be regarded as evidence in favor of a plate-like shape for the hemocyanin molecules.

*Limulus polyphemus Hemocyanin*—A preparation of *Limulus* hemocyanin, kindly provided by Dr. W. C. Boyd, was mounted at a concentration of  $10^{-6}$  gm. per cc. in water. Twenty of the particles shown in a typical micrograph (Fig. 5) have diameters of approximately 20  $m\mu$ , and the average diameter of the thirty-three particles which are shown was estimated to be 22  $m\mu$ . The large particle at the upper right appears to have a regular circumference, but nevertheless possesses three centers of density and is therefore definitely resolvable into three particles. Eriksson-Quensel and Svedberg found a preparation of *Limulus* hemocyanin to have four components possessing sedimentation constants of 56.5, 34.6, 16.1, and 5.9, respectively (12). A portion of the preparation used in the present study was examined in the analytical ultracentrifuge and found to contain but two components. The fraction comprising about 10 per cent of the preparation had a sedimentation constant of 87.3, and the fraction comprising about 90 per cent of the material had a sedimentation constant of 59.9. On the basis of a spherical shape, the latter or major component may be calculated to have a particle diameter of about 19  $m\mu$ . The direct and indirect estimations of molecular size are therefore in good agreement. The molecules shown in Fig. 5 are definitely thicker than those of the other two hemocyanins. The results indicate that the molecules of *Limulus* hemocyanin are probably essentially spherical in shape and are about 20  $m\mu$  in diameter.

*Viviparus malleatus Hemocyanin*—A preparation of the hemocyanin, kindly supplied by Dr. W. C. Boyd, was mounted at a concentration of  $10^{-6}$  gm. per cc. in water. A typical micrograph is shown in Fig. 6. About thirty-two very faintly appearing particles, whose average diameter is about 29  $m\mu$ , may be seen. Eighteen of these particles are about 36  $m\mu$  in diameter. The faint appearance of the particles indicates that they are thin; hence it is probable that they have a disk- or plate-like shape.

The sedimentation or diffusion constant of this hemocyanin does not appear to have been determined. Therefore, a portion of the preparation used for the electron micrographs was studied in the analytical ultracentrifuge by Dr. M. A. Lauffer. A solution containing 3.1 mg. of protein per cc. in 0.1 M phosphate buffer at pH 7 was centrifuged in a 6 mm. cell at

22,200 R.P.M. at 22.5°. A small amount of material was practically un-sedimentable at the speed used and hence consisted of material of low molecular weight beyond the range of the electron microscope. The majority of the material comprised a single component which possessed a sedimentation constant,  $S_{20w}$ , of 95.0. On the basis of a spherical shape, this material should have a particle diameter of about 24 m $\mu$ . However, the diameters of most of the molecules shown in Fig. 6 are considerably larger than this value. This discrepancy, as well as the faint appearance of the particles in the micrograph, would be explainable if the dried molecules were assumed to have plate-like rather than spherical shapes.

#### DISCUSSION

The results indicate that the electron microscope is very useful in the elucidation of the sizes and particularly the shapes of the larger protein molecules. In the case of molecules which are essentially spherical in shape, such as, for example, bushy stunt virus and probably also *Limulus* hemocyanin, the agreement between molecular sizes estimated by indirect methods and those estimated directly from electron micrographs is excellent. The fact that in one and perhaps two cases the molecular sizes estimated by means of the electron microscope are not in accord with those estimated by indirect methods based on the assumption of a spherical shape is a probable indication that the latter assumption is not justified. Discrepancies between molecular weights estimated by different indirect methods are usually regarded as being due to hydration, to asymmetry, or to both, and in most cases it is not possible to determine by the indirect methods which factor is the more important. For the larger protein molecules it is now possible to determine directly by means of the electron microscope the cases of molecular asymmetry due to a rod-like shape, such as occurs, for example, in tobacco mosaic virus (9), and it is possible to secure some measure of information regarding disk- or plate-like shapes, such as appear to occur in *Viviparus* hemocyanin and probably also in *Busycon* hemocyanin. This type of information, which can be provided by electron micrographs, should make it possible to evaluate better the relative importance of hydration and asymmetry in molecular weight estimations of the larger protein molecules.

The pictures obtained here without a limiting objective aperture are quite comparable in definition to those obtained for analogous materials by von Ardenne using a small objective aperture (11). Apparently the spherical aberration of the lens itself forms an excellent natural aperture, free from dirt, naturally centered, and having the additional advantages of easier alignment of the microscope, easier focusing, and making more detail visible on the fluorescent screen for thicker specimens.

## SUMMARY

Preparations of five different proteins and of a mixture of tobacco mosaic virus and silver particles were examined by means of an electron microscope. Some of the silver particles shown in the micrograph were estimated to have diameters of about 15 Å. The molecules of bushy stunt virus and of *Limulus polyphemus* hemocyanin appeared to be essentially spherical in shape and to possess diameters of approximately 26 and 20 mμ, respectively. Micrographs of *Busycon canaliculatum* and *Viviparus malleatus* hemocyanins showed particles having average diameters of about 22 and 29 mμ, respectively. From the low contrast of these particles in the electron microscope and from a consideration of ultracentrifuge data, it is inferred that the molecules of these two hemocyanins are probably plate-like in shape. The micrograph of edestin showed many particles ranging up to about 11 mμ in diameter and an almost complete absence of larger particles.

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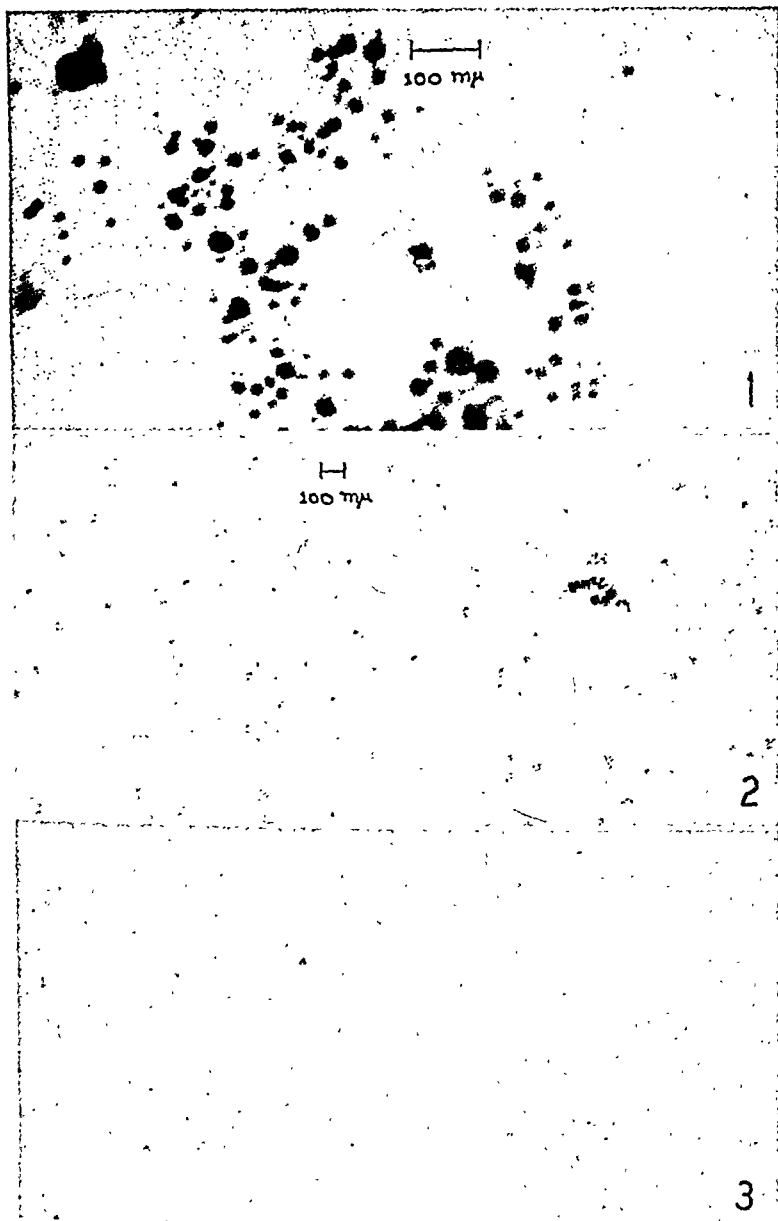
## EXPLANATION OF PLATES

## PLATE 1

FIG. 1. Electron micrograph of tobacco mosaic virus with minute silver particles. The latter can be detected down to diameters of about 15 Å.  $\times 100,000$ . All pictures were taken with 60 kilovolt-electrons.

FIG. 2. Electron micrograph showing spherical molecules of bushy stunt virus about 26 mμ in diameter.  $\times 35,600$ .

FIG. 3. Electron micrograph of commercial edestin showing particles most of which range up to about 11 mμ in diameter.  $\times 35,600$ .




(Stanley and Anderson: Electron micrographs of protein molecules)

## PLATE 2

FIG. 4. Electron micrograph showing molecules of *Busycon canaliculatum* hemocyanin, most of which have a diameter of about 22  $m\mu$ .  $\times 48,000$ . The low contrast of the particles in comparison with those of bushy stunt virus indicates a disk-like shape.

FIG. 5. Electron micrograph of *Limulus polyphemus* hemocyanin showing essentially spherical molecules having a diameter of about 20  $m\mu$ .  $\times 48,000$ .

FIG. 6. Electron micrograph showing disk-shaped molecules of *Viviparus malacatus* hemocyanin about 29  $m\mu$  in diameter.  $\times 48,000$ .

  
100m $\mu$

4

5

6

(Stanley and Anderson: Electron micrographs of protein molecules)





# THE NATURE OF THE GLYCEROPHOSPHORIC ACID PRESENT IN PHOSPHATIDES

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Early work of Willstätter and Ludecke (1) on glycerophosphoric acid from egg lecithin and of Levene and Rolf (2) on glycerophosphoric acid prepared both from brain lecithin and from brain cephalin seemed to establish that glycerophosphoric acid from all three sources was optically active and essentially the same and therefore was the  $\alpha$  form. On the other hand Bailly (3) could not find any optical activity in glycerophosphoric acid carefully isolated from different phosphatides. Karrer and Salomon (4), making use of the fact that barium forms a double salt with nitric acid and  $\beta$ -glycerophosphoric acid but not with  $\alpha$ -glycerophosphoric acid, showed that glycerophosphoric acid from phosphatides was a mixture of both  $\alpha$  and  $\beta$  forms. Although they found  $\alpha_D^{20} = -0.40^\circ$  for the mixture of the two forms, the  $\alpha$  form isolated from the mixture failed to show any optical activity. Therefore, Karrer and Salomon attributed to impurities the optical activity of the mixture.

Fleury and Lange (5), in 1933, provided a simple and accurate method for the estimation of  $\alpha$ -glycerophosphoric acid in mixtures of  $\alpha$  and  $\beta$  forms by the use of  $\text{HIO}_4$ , then recently shown by Malaprade (6) to be a specific reagent for polyalcohols having the  $-\text{CH}(\text{OH})-\text{CH}(\text{OH})-$  group. Fleury and Lange found in accordance with this principle that  $\alpha$ -glycerophosphoric acid reacted in a few minutes, a mole of  $\text{HIO}_4$  being used per mole of glycerophosphoric acid, while  $\beta$ -glycerophosphoric acid was completely stable under the same conditions. Previously to this work, Bailly and Gaumé (7) had been accumulating evidence to the effect that in methyl esters of glycerophosphoric acid, the phosphoryl radical migrated from the  $\alpha$  to the  $\beta$  position on the glycerol carbon chain, under a series of treatments that fell short of actual hydrolysis. Later M. C. Bailly (8) showed that  $\beta$ -glycerophosphoric acid can be quantitatively changed to  $\alpha$ -glycerophosphoric acid by treatment with boiling acid under certain conditions. She showed also (9) that this is a reversible change and that the point of equilibrium between the  $\alpha$  and  $\beta$  forms depends on conditions of treatment: treatment with alkali brings a predominance of the  $\beta$  form and acid treatment brings a predominance of the  $\alpha$  form. Her results have been confirmed by Verkade *et al.* (10). Obviously these findings make it impossible to ascertain in which form glycerophosphoric acid is present in

phosphatides by study of the glycerophosphoric acid left after the fatty acids are hydrolyzed off with acid or alkali.

In the course of work on the chemistry of phosphatides isolated from the cephalin fraction of brain lipids the writer has been brought to reconsider the nature of glycerophosphoric acid present in these phosphatides. Barium glycerophosphate has been prepared, by a procedure described elsewhere (11), from three different components of brain "cephalin;" namely, an inositol-containing phosphatide fraction (12), phosphatidyl serine (13), and phosphatidyl ethanolamine (11). The method of preparation involved boiling of the phosphatides 30 minutes with 6 *N* HCl to hydrolyze off the fatty acids and nitrogenous constituents, leaving the glycerophosphoric acid intact. Glycerophosphoric acid prepared from the three different fractions was analyzed for  $\alpha$ -glycerophosphoric acid by the  $\text{HIO}_4$  method of Fleury and Lange (5) and found to consist in all three cases of about 73 per cent  $\alpha$  form and about 27 per cent  $\beta$  form. Barium glycerophosphate prepared from all three sources in concentrations up to 15 per cent has been found optically inactive. Furthermore pure  $\beta$ -glycerophosphoric acid and  $\alpha$ -glycerophosphoric acid (prepared from the  $\beta$  form by M. C. Bailly's method (8)) have been submitted separately to the acid hydrolysis used for the isolation of glycerophosphates from phosphatides and in both cases the same mixture of 73 per cent  $\alpha$  form and 27 per cent  $\beta$  form was yielded, regardless of which isomer was present at the start. Finally glycerophosphoric acid has been prepared from phosphatidyl serine (13) with cold barium hydroxide as a hydrolyzing agent instead of HCl. The glycerophosphate obtained has been found to be 80 per cent  $\beta$  form and optically inactive.

The lack of optical activity of glycerophosphoric acid prepared from phosphatides is to be expected, since migration of the phosphoryl radical must result in a racemic product. Our results on this point confirm those of Bailly (3) and Karrer and Salomon (4). Such slight optical activity as has been found in samples of glycerophosphoric acid from phosphatides by other workers (1, 2) is probably due to the presence of small amounts of optically active impurities. Unfortunately in most cases glycerophosphoric acid has been identified as the barium salt merely by a barium analysis, and it is therefore difficult to ascertain how pure most preparations were.

#### SUMMARY

The methods of isolation hitherto used to prepare glycerophosphoric acid from phosphatides hydrolyzed with alkali or acid yield optically inactive mixtures of  $\alpha$ - and  $\beta$ -glycerophosphoric acids, in proportions which depend on the conditions of hydrolysis. Hence available data do not provide

evidence as to whether the glycerophosphoric acid in phosphatides is the  $\alpha$  or the  $\beta$  form.

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# BRAIN CEPHALIN, A MIXTURE OF PHOSPHATIDES. SEPARATION FROM IT OF PHOSPHATIDYL SERINE, PHOSPHATIDYL ETHANOLAMINE, AND A FRACTION CONTAINING AN INOSITOL PHOSPHATIDE\*

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The phosphatide fraction from brain called "cephalin" (1) was formerly accepted as a definite compound of glycerophosphoric acid with 2 molecules of fatty acids and 1 of ethanolamine. Folch and Schneider (2) have recently shown, however, that cephalin prepared by standard methods has 40 to 70 per cent of its nitrogenous constituent not in ethanolamine but in *l*(+)-serine. A phosphatide has been separated from cephalin that has all of its nitrogen as a  $\beta$ -hydroxyamino acid and the latter has been isolated as pure *l*(+)-serine. This phosphatide has been given the name of *phosphatidyl serine* (3).

In a recent note (4) it has been shown that brain "cephalin" contains still other phosphatides, and that part of it consists of one or more phosphatides containing inositol.

The present paper contains detailed proof of this statement and description of a method for separation from cephalin of three different fractions. The method is based on the fact that the individual phosphatides, the mixture of which is called brain cephalin, exhibit marked differences in their respective solubilities in mixtures of chloroform and alcohol. The separation was achieved by adding to a chloroform solution of brain cephalin increasing amounts of alcohol and collecting separately material precipitated at different concentrations of alcohol in the mixture. Cephalin prepared from brain by the classical methods has thereby been separated into three fractions: (a) *phosphatidyl ethanolamine* so called because it has its nitrogen as ethanolamine and its P as glycerophosphoric acid; it appears to have the composition previously attributed to the entire cephalin; (b) *phosphatidyl serine*; and (c) a mixture of phosphatides of which at least one contains inositol.

The inositol-containing fraction itself is a mixture characterized, as compared with *phosphatidyl ethanolamine* and *phosphatidyl serine*, by relatively low solubility in alcohol, low carbon content, high phosphorus content and P:N ratio, and the presence of inositol. Part of the nitrogen

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is in the form of serine, and there is glycerol as well as inositol. Of serine and glycerol, one or both may be due partly or entirely to the presence of phosphatidyl serine mixed with the inositol phosphatide.

*Phosphatidyl ethanolamine*, probably isolated in a relative state of purity for the first time, is freely soluble in alcohol. Its constituent fatty acids are different from those in phosphatidyl serine as shown by its iodine number of 78 as compared to 33 for phosphatidyl serine. As isolated from brain without acid treatment, it is free of ash, while phosphatidyl serine thus isolated is a salt, mostly of potassium. It seems desirable to call this substance phosphatidyl ethanolamine rather than cephalin, because the latter name has for decades been applied to a mixture of different composition, and of different properties. Thus "cephalin" indicates a phosphatide mixture insoluble in alcohol, whereas the separated phosphatidyl ethanolamine is freely soluble in alcohol.

That cephalin which appears to be such a crude mixture has not been fractionated before is probably due to the fact that it has been customary to free cephalin from water-soluble impurities by treatment with HCl. This treatment removes potassium and sodium with which the acidic phosphatides, namely phosphatidyl serine and most of the constituents of the mixture referred to as inositol phosphatide fraction, are combined, and leaves all three cephalin fractions in the form of free acids. This mixture is difficult to fractionate. Our methods of fractionation have succeeded partly because, of the initial cephalin mixture used, the phosphatidyl ethanolamine was present as the free ampholyte, while the other two fractions were in the form of potassium and sodium salts.

That phosphatidyl ethanolamine is not the result of postmortem decarboxylation of phosphatidyl serine was shown by the fact that cephalin prepared from brain removed from an anesthetized dog and minced immediately with acetone cooled at  $-72^{\circ}$  contained the same amounts of ethanolamine and of serine as cephalin prepared in the usual way.

#### EXPERIMENTAL

*Analytical Methods*—Manometric methods were routinely used for the estimation of C (5), P (6), N (7),  $\text{NH}_2\text{-N}$  (8), and carboxyl N (9). Inositol<sup>1</sup> was estimated by Woolley's microbiological method (10), iodine numbers by Yasuda's method (11), and glycerol by the Blix method (12). Barium was estimated by weighing it as  $\text{BaSO}_4$ .

In some cases in which elementary composition has been used for identification of compounds, C and H were estimated by dry combustion, lead chromate being used in compounds that had ash, and N by the Dumas method. It has been found that C values by the wet combustion method

<sup>1</sup> Inositol analyses were carried out by Dr. D. W. Woolley.

of Van Slyke and Folch (5) agreed with those obtained by dry combustion. In the case of some lipid fractions it was also found that values for total N estimated by the Dumas method were about 1.01 times those obtained by manometric micro-Kjeldahl determination (7).

Ashing was carried out by heating a weighed sample of material in a silica crucible at 500° in an electric furnace to constant weight. Overnight heating has been found sufficient. On some ashes, estimations of Ca (13), Mg (14), K (15), and Na (16) were carried out.<sup>2</sup>

*Preparation of Brain Cephalin*—By introducing into the preparation of brain cephalin the use of a mechanical mincer, the use of dialysis to remove water-soluble impurities, and the use of freezing and drying in a high vacuum as a means of removing water, it has been possible to obtain cephalin from brain with a higher yield, in a shorter time, and by a milder treatment than before.

Fresh ox brains are freed of membranes and the tissue minced with acetone in a Waring blender. About 100 gm. of tissue at a time are introduced into the cup, the blades set in motion, and 300 cc. of acetone added slowly. The mincing is let go on for about 2 minutes. After the entire material has been minced, the portions are combined and acetone is added so that there are at least 3.8 cc. of acetone per gm. of tissue. With less acetone the separation of acetone-insoluble material takes place very slowly or not at all. With more than 3.8 cc. per gm. of tissue, on the other hand, the acetone-insoluble material precipitates out very quickly and filtration can be started within a few minutes.

The acetone is filtered off and discarded. The precipitate is extracted in succession, a second time with acetone, once with alcohol, and twice with petroleum ether (b.p. 30–60°), some 4 cc. of each solvent being used per gm. of original tissue. The two petroleum ether extracts are combined and concentrated to dryness by vacuum distillation. It is important to carry the removal of petroleum ether to completion. The residue is then dissolved in ethyl ether, some 200 cc. of ethyl ether being used for each kilo of original tissue. Only part of the residue is soluble in ether, but all of it will go into suspension; at this point the suspension would resist centrifugation. However, if it is transferred to a glass-stoppered cylinder and let stand in the ice box, the suspended material starts to settle out after a day or two. When a clear supernatant solution appears in the upper quarter of the cylinder, the suspension can be separated by centrifugation. It is then spun down and washed twice with cold ether, the washings being added to the ether extract.

The combined ether extracts are concentrated to dryness by vacuum distillation at room temperature and the residue is dissolved in ether, 50 cc. being used for each kilo of initial brain tissue. The ethereal solution is let stand in a cylinder in the ice box overnight. Any precipitate that forms is discarded. The ether solution is diluted with an equal volume of ether, and 5 volumes of alcohol are added slowly with stirring to precipitate the cephalin. The mixture is let stand at room temperature until a clear supernatant is formed, which usually takes less than an hour. The crude cephalin precipitate is collected on a Buchner filter and suspended in acetone, 100

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<sup>2</sup> Ca and Mg estimations were made by Dr. K. Emerson, Jr. K and Na estimations were made by Dr. W. W. Beckman.



cc. being used per kilo of original brain tissue. The suspension is shaken 40 minutes to dissolve acetone-soluble impurities. The clear supernatant is discarded, fresh acetone added to the precipitate, and the procedure repeated. The cephalin powder is then collected on a Buchner filter and dried.

The product is a tan powder. The yield is about 15 gm. per kilo of initial tissue. A typical preparation on analysis yields C 55.2, P 4.13, N 1.59,  $\text{NH}_2\text{-N}$  1.51, carboxyl N 0.76 per cent,  $\text{NH}_2\text{-N:N}$  0.96, P:N 1.17.

*Fractionation of Brain Cephalin by the Chloroform-Alcohol Method*—The procedure must be applied to cephalin that has not been treated with dilute HCl.

1 gm. of the above preparation of cephalin is dissolved in 8 cc. of  $\text{CHCl}_3$  and to the clear solution are added 9.1 cc. of absolute alcohol (1.135 as much alcohol as chloroform by volume). A turbidity develops and on standing or by centrifugation the mixture resolves itself into a viscous underlying layer (Fraction I) and a clear supernatant. The supernatant is decanted and mixed with 2.7 cc. of alcohol. From the alcoholic mixture a second underlying layer separates out (Fraction II). The supernatant is decanted and to it are added 25 cc. of alcohol. A precipitate appears which is collected on a Buchner filter (Fraction III) and dried. The filtrate is concentrated to half its volume in a vacuum and let stand in the ice box for 2 or 3 days. A precipitate that forms (Fraction IV) is separated by filtration in the cold and dried. The filtrate is concentrated to 1 cc. and to it 5 cc. of acetone are added. After the mixture has stood in the ice box for 1 day, an acetone-insoluble material is precipitated (Fraction V). It is filtered and dried. To the viscous solutions that constitute Fractions I and II, alcohol is added. Solid precipitates are formed that can be collected on Buchner filters and dried.

All five fractions are then freed of water-soluble impurities by dialysis. Of each fraction a 3 per cent aqueous emulsion is prepared by adding 30 cc. of water per gm. of material and shaking until homogeneous. The emulsion is then transferred to a cellophane casing and dialyzed at 4° against distilled water for 4 days with several changes of the outside liquid. After this the emulsion is transferred to a round bottom flask, frozen by partly immersing the flask in a mixture of alcohol and solid  $\text{CO}_2$ , and while frozen dried in a high vacuum (0.1 mm. of Hg or better). The product is a white powder too fluffy to be handled conveniently. To remedy this, the material is suspended in acetone, collected on a Buchner filter, and dried.

*Analysis of Fractions Obtained from Brain Cephalin by Chloroform-Alcohol Method*—The results of chemical analysis of the different fractions are tabulated in Table I. From inspection of the results it is seen that Fractions II and IV, which together account for less than one-fourth of the parental cephalin, are mixtures of fractions adjoining them. On the other hand, Fractions I, III, and V exhibit striking differences in composition. Fraction I contains inositol, which is absent from Fractions III and V. Fraction III is phosphatidyl serine having over 90 per cent of its nitrogen as amino acid nitrogen. Fraction V, phosphatidyl ethanolamine, has practically no amino acid nitrogen and on further study is shown to have most of its nitrogen as ethanolamine.

## FRACTION CONTAINING INOSITOL PHOSPHATIDE

This fraction appears as a loose white powder that may or may not acquire a slight tan color on standing *in vacuo* over calcium chloride or on being recovered from solutions in organic solvents. Over calcium chloride in an evacuated desiccator at room temperature it retains about 1.6 per cent water which it loses at 80° and regains when stored away in the desiccator, even *in vacuo*.

The chemical composition is given in Table I. The large amount of ash probably indicates that a large part of the phosphatide material is strongly acidic in character.

TABLE I

*Analysis of Fractions Isolated from Brain Cephalin by Chloroform-Alcohol Method*

Components	Fraction I (inositol phosphatide)	Fraction II	Fraction III (phosphatidyl serine)	Fraction IV	Fraction V (phosphatidyl ethanolamine)
(1)	(2)	(3)	(4)	(5)	(6)
	per cent	per cent	per cent	per cent	per cent
C.....	55.0	59.0	60.2	63.0	66.1
P.....	4.25	3.86	3.58	3.60	3.65
N.....	1.15	1.36	1.62	1.75	1.78
Amino N*.....	1.15	1.36	1.64	1.60	1.50
Carboxyl N†.....	0.70	0.80	1.47	0.60	<0.02
Inositol.....	6.8	3.4	<0.20	<0.20	<0.20
Iodine No.....	65.0		39.8		78.0
Ash.....	16.7		12.8		2.5
Yield in gm. per 100 gm. cephalin.	22.0	10.0	27.0	8.0	15.0

\* By the nitrous acid manometric method (8).

† By the ninhydrin-CO<sub>2</sub> method of Van Slyke, Dillon, MacFadyen, and Hamilton (9).

Among the products of hydrolysis inositol, glycerophosphoric acid, and serine have been isolated.

*Inositol*—The isolation of inositol has already been described (4). The amount isolated was 60 per cent of the amount found by analysis (Column 2, Table I).

#### *Isolation of Glycerophosphoric Acid from Inositol Phosphatide Fraction*

To remove ash, 4 gm. of Fraction I (Table I) were emulsified with 200 cc. of water and 50 cc. of 6 N HCl were added to the emulsion. The precipitate that was formed was spun down, washed once with N HCl, and hydrolyzed with 6 N HCl for 40 minutes under a reflux. This treatment hydrolyzes off nitrogenous constituents and fatty acids, but leaves phosphoric acid bound to glycerol. Whether this treatment splits

$\text{PO}_4$  from inositol is uncertain. After cooling, the fatty acids were filtered off, and the filtrate concentrated to dryness by vacuum distillation. The dry residue was taken up in water, and the solution decolorized with ash-free charcoal and concentrated to a volume of about 4 cc. To this 36 cc. of alcohol were added and the whole let stand overnight in the ice box to precipitate material other than glycerophosphoric acid. The next day the precipitate was spun down and the clear supernatant collected and concentrated to dryness by vacuum distillation. The residue was dissolved in water and  $\text{Ag}_2\text{O}$  and acetic acid were added to the solution to remove  $\text{Cl}^+$  and  $\text{PO}_4^{+++}$ . The excess Ag was removed by  $\text{H}_2\text{S}$ , the filtrate concentrated to dryness by vacuum distillation, and the residue dissolved in water. To precipitate glycerophosphoric acid neutral lead acetate (25 per cent aqueous solution) was added until no more precipitate formed. The lead glycerophosphate was washed with water by centrifugation. It was then suspended in water, and decomposed with  $\text{H}_2\text{S}$ , the glycerophosphoric acid passing into solution. The filtered solution was concentrated *in vacuo*, brought to about 15 cc., and alkalinized to pH 10 by addition of saturated aqueous  $\text{Ba}(\text{OH})_2$  solution. An equal volume of alcohol was added and the solution was let stand overnight in the ice box to precipitate barium glycerophosphate. The latter was spun down, washed twice with cold 50 per cent alcohol, and dried at  $140^\circ$  in a vacuum to constant weight.

The product weighed 510 mg. and on analysis proved to be barium glycerophosphate. It was free of inositol and of nitrogen. It accounted for 30 per cent of the phosphorus present in the starting material. The results were as follows:

$\text{C}_3\text{H}_7\text{O}_6\text{PBa}$ .	Calculated.	C 11.73,	P 10.1,	Ba 44.5,	glycerol 29.2
	Found.	" 11.61,	" 9.95,	" 45.3,	" 27.7

#### *Isolation of Serine from Inositol Phosphatide Fraction*

To remove ash, 3.5 gm. of Fraction I (Table I) were emulsified with 150 cc. of water and acidified with 12 cc. of concentrated  $\text{HCl}$ . A precipitate of the inositol-containing fraction formed and was spun down, washed once with  $\text{N HCl}$ , and hydrolyzed with boiling 6  $\text{N HCl}$  for 30 minutes under a reflux. After cooling, the fatty acids were filtered off. The filtrate was concentrated to dryness by vacuum distillation. The residue was dissolved in 15 cc. of water and decolorized with ash-free charcoal. To precipitate the serine, 1.1 gm. of *p*-hydroxyazobenzene-*p*-sulfonic acid,<sup>3</sup> a reagent introduced into amino acid chemistry by Stein, Moore, Stamm, Chou, and Bergmann (17), were added and dissolved with heat. The solution was let stand in the ice box for 2 days. Crystals appeared which were spun down in the cold and washed three times with 4 cc. portions of cold water. The dry crystals weighed 318 mg.

The supernatant and the washings were combined, concentrated to a volume of 6 cc., and let stand in the ice box. A second crop of crystals was thus obtained. They were collected and washed twice with cold water. They weighed 198 mg.

On analysis both crops proved to be serine *p*-hydroxyazobenzene-*p*-sulfonate. They accounted for 76.5 per cent of carboxyl N in the starting material. The results are given in Table II.

<sup>3</sup> *p*-Hydroxyazobenzene-*p*-sulfonic acid was provided by the kindness of Dr. Max Bergmann.

## PHOSPHATIDYL ETHANOLAMINE

The preparation was a slightly sticky white powder. On standing in a vacuum in the dark in a desiccator it acquired in a fortnight a tan color that turned later into a deep brown. With the change in color there was a progressive increase in stickiness which made the material difficult to handle. The change in physical appearance was not accompanied by any observable change in elementary composition.

The material, either fresh or colored, retained 1.7 per cent water. Drying at 80° in a vacuum removed this water, but when the material was replaced in a desiccator over calcium chloride it regained its former weight even *in vacuo*.

As seen from Table I, the composition accords with the formula assigned to cephalin in classical biochemistry. The absence of ash is to be expected from a compound having both an acidic and a basic group free. Among

TABLE II

*Analysis of Serine p-Hydroxyazobenzene-p-sulfonate Isolated from Fraction Containing Inositol Phosphatide*

Components	Found for 1st crop of crystals	Found for 2nd crop of crystals	Calculated for serine <i>p</i> -hydroxyazo- benzene- <i>p</i> -sulfo- nate (C <sub>18</sub> H <sub>17</sub> O <sub>7</sub> N <sub>3</sub> S)
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C.....	46.8	46.9	47.0
H.....	4.37	4.37	4.44
NH <sub>2</sub> -N.....	3.71	3.68	3.66
Carboxyl N ( <i>cf.</i> (9)).....	3.71	3.69	3.66
Ash.....	0.09	0.10	0.00

its cleavage products ethanolamine and glycerophosphoric acid have been isolated with high yields. Its iodine number is 78, which shows the presence of two double bonds for each atom of P. Therefore its fatty acids are different from those in phosphatidyl serine.

All the analytical figures in Table I (Column 6) were determined by direct analysis of the phosphatide or its ash, except the amino nitrogen determined by the nitrous acid method. Using water emulsions of phosphatidyl ethanolamine, we obtained results which were low and inconsistent, presumably because the emulsion coagulated in the nitrous acid mixture and became partly inaccessible to the reagent. Consequently, to determine the NH<sub>2</sub> nitrogen, the phosphatide was hydrolyzed for 4 hours with boiling 4 N HCl under a reflux. After cooling, the mixture was made up to volume and filtered free of separated fatty acids. The amino nitrogen was determined (8) on aliquot portions which were neutralized before the analysis.

*Isolation of Ethanolamine from Phosphatidyl Ethanolamine*

To remove ash and water-soluble impurities, 4.5 gm. of Fraction V (Table I) were emulsified with 300 cc. of water and 25 cc. of concentrated HCl were added to the emulsion. The precipitate that formed was spun down, washed once with  $\pi$  HCl, and hydrolyzed with boiling 4  $\pi$  HCl for 5 hours under a reflux. After cooling, the hydrolysate was filtered to remove the fatty acids and the filtrate concentrated to dryness by vacuum distillation. The residue was taken up in 25 cc. of water, the solution decolorized with ash-free charcoal, and 2.5 gm. of *p*-hydroxyazobenzene-*p*-sulfonic acid,<sup>3</sup> a reagent for ethanolamine introduced by Stein, Moore, Stamm, Chou, and Bergmann,<sup>4</sup> added to it and dissolved with heat. This reagent forms insoluble salts both with serine and ethanolamine. It could be used in this case to precipitate ethanolamine for analysis, because the amount of serine present was shown by carboxyl N (9) determination to be negligible. The solution was let stand overnight in the ice box. Crystals that formed were spun down in the cold and washed three times with 7 cc. portions of cold water. The dry crystals weighed 870 mg.

The supernatant and the washings were combined, concentrated to a volume of 8 cc., and let crystallize overnight in the ice box. The second crop of crystals was spun

TABLE III

*Analysis of Ethanolamine p-Hydroxyazobenzene-p-sulfonate Isolated from Phosphatidyl Ethanolamine*

Components	Found for 1st crop of crystals	Found for 2nd crop of crystals	Calculated for ethanolamine <i>p</i> - hydroxyazobenzene- <i>p</i> -sulfonate (C <sub>12</sub> H <sub>11</sub> O <sub>3</sub> N <sub>2</sub> S)
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C.....	49.37	49.42	49.55
H.....	5.20	5.22	5.19
NH <sub>2</sub> -N.....	4.07	4.10	4.13
Carboxyl N.....	0.00	0.00	0.00
Ash.....	0.12	0.15	0.00

down in the cold and washed twice with 2 cc. each time of cold water. The dry crystals weighed 368 mg.

On analysis both crops of crystals proved to be ethanolamine *p*-hydroxyazobenzene-*p*-sulfonate. They accounted for 74 per cent of NH<sub>2</sub>-N in the starting material. The results are given in Table III.

*Isolation of Glycerophosphoric Acid from Phosphatidyl Ethanolamine*

To remove ash, 2 gm. of Fraction V (Table I) were emulsified in 150 cc. of water and 10 cc. of concentrated HCl were added to the emulsion. The precipitate that formed was spun down, washed once with 0.6  $\pi$  HCl, and boiled for 30 minutes with 6  $\pi$  HCl under a reflux to split off the ethanolamine and fatty acids from the glycerophosphoric acid. After cooling, the fatty acids were filtered off, the filtrate concentrated to dryness by vacuum distillation, and the residue taken up in 25 cc. of water. To

<sup>4</sup> Stein, W. H., Moore, S., Stamm, G., Chou, C. Y., and Bergmann, M., unpublished results.

the solution were added 0.5 cc. of concentrated acetic acid and 1.5 gm. of  $\text{Ag}_2\text{O}$  to remove  $\text{Cl}^+$  and  $\text{PO}_4^{+++}$ . From here on the procedure followed was exactly that described for the isolation of glycerophosphoric acid from the inositol phosphatide fraction.

The material obtained weighed 437 mg. On analysis it proved to be barium glycerophosphate. It was free of N. It accounted for 61 per cent of P in the starting material. The results were as follows:

$\text{C}_8\text{H}_{17}\text{O}_6\text{PBa}$ .	Calculated.	C 11.73,	P 10.1,	Ba 44.5,	glycerol 29.2
	Found.	" 11.68,	" 10.1,	" 44.5,	" 28.3

The writer is indebted to Dr. D. D. Van Slyke for constant advice and helpful criticism.

#### SUMMARY

1. The "cephalin" fraction of brain phosphatides, formerly accepted as a definite compound, is shown to be a mixture of phosphatides. The individual phosphatides in the mixture exhibit differences in their respective solubilities in mixtures of chloroform and alcohol. This fact is made use of for the separation from cephalin of three different fractions; namely, (a) phosphatidyl serine (3), (b) a compound which has the composition formerly attributed to the whole cephalin, and which is called *phosphatidyl ethanolamine*, and (c) a mixture of phosphatides one or more of which contain inositol as a constituent (4).

2. The fraction containing inositol phosphatide is less soluble in alcohol than either phosphatidyl serine or phosphatidyl ethanolamine; from it have been isolated, besides inositol, glycerophosphoric acid and serine, the presence of which indicates the probable presence of phosphatidyl serine in the mixture.

3. Phosphatidyl ethanolamine, unlike the cephalin to which its composition was formerly assigned, is freely soluble in alcohol. From it have been isolated ethanolamine and glycerophosphoric acid.

4. With the exception of phosphatidyl ethanolamine, the phosphatides in the cephalin fraction of brain lipids are strongly acidic in character and are isolated from brain as salts of potassium and sodium when treatment with mineral acid is avoided in the isolation. Treatment with  $\text{HCl}$  removes the alkali cations. The method described for separating the phosphatides of cephalin is effective only when applied to material that has not had its mineral bases removed.

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# FAT METABOLISM IN THE LUNGS

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The importance of the blood in the transport of fat in the body is evident from the lipemia which normally accompanies fat absorption. The participation of the liver and the intestinal mucosa in the metabolism of fat, through the processes of desaturation and phosphorylation, is generally accepted (1). That the lungs, as well, participate in fat metabolism has been claimed by Hetenyi (2), who found that following the administration of olive oil to rabbits and dogs the fat content of the lungs was increased as much as 600 per cent. His interpretation was that fat absorbed from the small intestine reaches the lungs by way of the lymph channels, is temporarily detained there, and later released to the blood. On the other hand, Schrade (3) could find no change in the total blood fat content as the blood passes through the lung capillaries, either in the fasting state or during fat absorption. Sinclair (4) found that the incorporation of elaidic acid into the phospholipids of the lungs of rats fed a high elaidin diet was low compared to such organs as the small intestine, the liver, skeletal muscle, kidneys, heart, and red blood cells.

In the present study the problem of the participation of the lungs in fat metabolism has been subjected to investigation by determining changes in the lipid content of the lungs, first, during active fat absorption from the intestine, and second, during fasting, when there are rapid mobilization and utilization of stored fat.

## Methods

Male, 3 month-old, albino mice, previously maintained on a diet of oats and Purina dog chow, were used. After initial weighing the mice were placed in individual wire cages which had coarse screen bottoms, so that coprophagy was minimal. Water was supplied *ad libitum*. The temperature of the room was reasonably constant at about 25°. Food was withheld from the mice used in the fat absorption experiments for 10 to 12 hours to insure better consumption of the high fat diet. They were then fed ground Purina dog chow supplemented with 40 per cent lard, and sacrificed at intervals of 4 to 6½ hours later. Blood samples were obtained from the axillary artery according to the method described by Kuhn (5). The lungs were removed as quickly as possible, weighed, and placed in 95



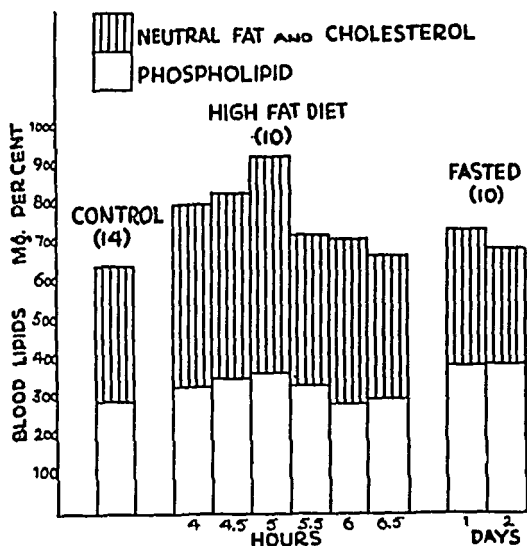


FIG. 1. The effect of a high fat diet and fasting on the blood lipids of mice. The figures in parentheses represent the number of animals used.

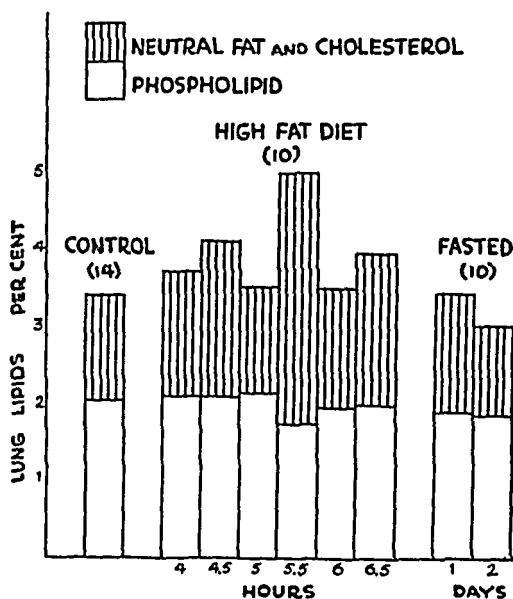


FIG. 2. The effect of a high fat diet and fasting on the lung lipids of mice. The figures in parentheses represent the number of animals used.

per cent alcohol. In the fasting experiments food was withheld from the mice for 1 and 2 day periods prior to sacrificing them. After the final weighing, blood samples were drawn by heart puncture and the lungs taken for analysis. The whole blood and the lung samples were analyzed by standard methods for phospholipid (6) and acetone-soluble lipid (neutral fat and cholesterol) (7), from which the total lipid values were calculated.

## RESULTS AND DISCUSSION

The lipemia which normally accompanies fat absorption from the intestine was found to reach its maximum 5 hours following the administration of the high fat diet (Fig. 1) and amounted to an increase of 43 per cent over the control level. While the blood phospholipid showed an increase of 24 per cent at this time, the rise in the total lipid content was due largely to an increase (58 per cent) in the neutral fat and cholesterol fraction.

The lipid content of the lungs of mice maintained on a standard diet varied from 2.96 to 4.10 per cent, with an average value of 3.46 per cent. The variation was confined mainly to the neutral fat and cholesterol fraction. Following the administration of a high fat diet, the total lipid content of the lungs was likewise variable, ranging from 3.24 to 5.05 per cent. However, no statistically significant increase over the control level was evident (Fig. 2). The phospholipid content of the lungs during this period was strikingly constant; the variation in total lipid was due chiefly to changes in the acetone-soluble fraction.

Mice fasted for 1 and 2 day periods, during which time they lost 15 to 20 per cent of their body weight, showed a moderate lipemia (Fig. 1) amounting to about 14 per cent on the 1st day. In contrast to the lipemia occurring in the fat-fed mice, the observed increase in the total lipid content of the blood was due mainly, if not entirely, to an increase (32 per cent) in the phospholipid fraction. These results are in agreement with those previously reported for fasted mice (8).

Fasting mice will completely deplete their fat reserves in 48 hours and at the same time show a marked increase in liver fat (9). During such a period of rapid mobilization and utilization of stored fat the lipids of the lungs failed to show any significant changes from normal (Fig. 2).

## SUMMARY

Male, 3 month-old, albino mice showed no significant changes from normal in the lipid content of the lungs, either during active absorption of fat from the intestine accompanied by a marked lipemia or during fasting for 1 and 2 day periods, when there are rapid mobilization and utilization

of stored fat. These observations do not support the conclusion that the lungs actively participate in fat metabolism.

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# THE PARTIAL PURIFICATION AND SOME OBSERVATIONS ON THE NATURE OF THE PARATHYROID HORMONE\*

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The most active preparation of the calcium-controlling principle of the parathyroid gland was, at the time this investigation was undertaken, that of Collip and Clark (1). This had been obtained by acid extraction of cattle glands followed by several isoelectric precipitations. The product, which had an activity of approximately 110 U.S.P.<sup>1</sup> units per mg. of nitrogen, behaved as a fairly well characterized protein, but there was no evidence for its chemical homogeneity. The material used by Tweedy and his collaborators in a series of investigations of the chemical nature of the hormone was obtained by a somewhat modified procedure, and in all examples reported (2-5) had an activity equal to about half that of Collip's earlier preparation. The physical nature of these various products, as well as their relatively low biological activity, suggested that a considerable purification should be possible if an appropriate method were found. Chemical study of such a preparation should yield valuable information regarding the nature of the active principle.

The present communication describes a procedure which provides a parathyroid hormone preparation having a potency of approximately 300 U.S.P. units per mg. of nitrogen. A number of observations regarding the physical and chemical properties of this product are also reported.

## *Method of Assay*

Any study of the parathyroid hormone is greatly handicapped by the unsatisfactory nature of the methods available for its biological assay. The standard procedure is that of Collip and Clark (6, 7) and depends upon the increase in total serum calcium of normal dogs after subcutaneous injection of the active principle. Since the response of dogs is variable, assays of a single sample must be made on a group of six to ten animals before reasonably significant values are obtained. Indeed Bliss and Rose

\* Supported by a grant from the Rockefeller Foundation.

<sup>1</sup> The U.S.P. unit is 0.01 of the amount of parathyroid hormone required to raise the serum calcium of a 10 to 12 kilo dog 1 mg. per cent within 16 hours after subcutaneous injection. This is 0.2 of the earlier Collip unit. We have adopted the term "nitrogen potency" to indicate the number of U.S.P. units contained in a given preparation per mg. of nitrogen.

in a statistical analysis of the problem (8) decided that an even greater number of animals was required to obtain a standard deviation of less than 10 per cent. Animals may not be used more frequently than once a week; so that a very simple fractionation of the hormone, from which four fractions are obtained, may tie up a group of eight animals for as long as a month.

To overcome this disadvantage various other assay procedures have been proposed. In the method of Hamilton and Schwartz (9) the serum calcium of rabbits is followed after the injection of parathyroid hormone and during the repeated oral administration of small quantities of calcium chloride. The response of rabbits, however, is too variable to give satisfactory results. Dyer studied the use of rabbits (10) and of mice (11), determining the increase in calcium excretion in the urine after injection of active extracts. Truszkowski and others (12) have recently modified the rat urinemethod, but it can be fairly stated that the fluctuation from day to day in the calcium excretion of these animals largely invalidates the procedures. The familiar dog serum calcium method is both more accurate and convenient than these alternate methods, and has the additional advantage that it has been widely employed in previous studies of the parathyroid hormone.

We have assembled in Table I the data obtained from a series of bioassays of two parathyroid preparations, for which twelve different animals were used. There appear to be two factors responsible for deviations from the average response: (1) the weight of the animal, which causes a decreasing response roughly proportional to increasing weight, and (2) the individual sensitivity of the animal, which, for instance, is responsible for the poor reactions of the dogs weighing 6.8 and 6.9 kilos. Both of these factors may be somewhat corrected for by standardizing the dogs and relating the response to that from the standard sample, as has been done in the last two columns of Table I.

Rather than report activities in terms of serum calcium increase per mg. of hormone per kilo of body weight, as is occasionally done (4, 5), we have adopted the simpler procedure of selecting a group of dogs of fairly uniform size and of reporting their response directly, irrespective of body weight. Successive assays with this group thus have considerable comparative significance.

The response of our assay animals to several different lots of parathyroid hormone is shown in Table II. Individual variations in response to a single dose may be found by comparison with the average in the last horizontal line. If we neglect the weight factor, all of the animals being roughly of the same size, it is possible to make deductions regarding the individual sensitivities of the animals. Dogs 1 to 4 were more consistent

TABLE I

*Assays of Two Parathyroid Preparations on Series of Twelve Dogs\**

Serum calcium values, estimated by the method of Clark and Collip (13), were determined before and 16 hours after injection. Solution A was an aqueous solution of a crude preparation and contained 12.6 mg. of nitrogen per ml.; Solution B contained 1.9 mg. of nitrogen per ml. of material obtained by ammonium sulfate fractionation.

Weight of dog	Response, increase in serum calcium per ml. solution		Response related to standard sample	
	Solution A	Solution B	Solution A	Solution B
kg.	mg. per cent	mg. per cent	units per ml.	units per ml.
6.8	1.10	1.00	180	170
6.9	1.90	2.00	170	180
9.2	3.50	2.45	340	240
9.9	2.55	3.20	260	320
11.3	2.90	2.45	260	220
12.3	2.20	2.25	200	200
13.0		1.50		140
14.4	1.40	1.50	140	160
15.8	1.30	1.35	140	140
16.8	1.20	1.15	220	210
16.9	1.50	1.20	200	170
17.9	0.65	1.10	130	220
Average.....	1.85	1.75	205	200

\* The authors are indebted to Dr. W. E. Bunney of the Biological Laboratories of E. R. Squibb and Sons for these assays.

TABLE II

*Characteristic Responses of Seven Dogs Employed in This Study*

The letters correspond to eight different preparations administered in varying dosage. Responses are given in terms of U.S.P. units; i.e., serum calcium rise  $\times 100$ .

Dog No.	Weight	Preparation							
		A*	B	C	D	E	F	G	H
	kg.								
1	13.4	480	280	390	550	400	380	200	490
2	13.2	490	200	240	480	420	610	350	330
3	14.8	380	210	190	330	640	590	180	710
4	13.0	390	260	320	310	520	520	180	690
5	19.2†	270	170	260	340	420		210	310
6	16.6	90	70	70	330	320	140	0	460
7	15.2	200	80	140	180	340	60	180	510
Average response to given dose.....		330	183	227	360	437	383	186	500

\* 400 units of commercial product (E. R. Squibb and Sons).

† Dog 5 tended to be edematous.

in their response; that of Dogs 5 to 7 was erratic, with a tendency to be quite low. Assays performed with these latter dogs must therefore be expected to be on the low side. All the assays in this paper are reported along with the number of the animal used so that this factor may be taken into consideration. Dogs 1 to 4 were Dalmatians of known background and Dogs 5 to 7 were mongrels; all were young males.

We have frequently used assays, carried out on two or three animals, to indicate for our own purposes trends in fractionation procedures, but in every case in which nitrogen potencies are reported, these have been obtained from a sufficient number of assays to be trustworthy.

Calcium was determined throughout this paper, with the exception of the experiments of Table I, by the procedure of Fiske and Logan (14).

### *Preparation*

The procedure finally adopted, as giving the most satisfactory yield, consists of three major steps. The first, extraction of the active principle, is largely that of Hanson (15) and will not be described in detail. The second involves ammonium sulfate fractionation at pH 6.0. The third procedure, benzoic acid adsorption, is adapted from that of Moloney and Findlay (16) who used it in concentrating insulin. The nitrogen potencies after application of the successive steps are 10 to 15, 60 to 100, and 250 to 325 units per mg. of nitrogen, respectively.

In addition to this procedure numerous other fractionation methods were investigated; the results of these are briefly reported.

*Extraction*—Acetone-dried and defatted glands are restored to their original weight by the addition of water, and are then extracted for 30 minutes at 100° with 0.5 volume of 3 per cent hydrochloric acid. After cooling, inert protein is precipitated at pH 4.0 by the addition of alcohol to 80 per cent by volume. The active principle is then obtained by precipitation with ether from the alcoholic solution and drying with acetone. About 250 gm. of gray powder containing 11 to 13 per cent of nitrogen are obtained from 15 pounds of fresh glands.

*Ammonium Sulfate Fractionation*—A solution of 50 gm. of the above preparation in 400 ml. of water is adjusted to pH 8.0 with ammonium hydroxide and is diluted by the slow addition of an equal volume of 2.5 M ammonium sulfate, with mechanical stirring. Adjustment of the pH to 5.9 to 6.0 with 1 M sulfuric acid, with a glass electrode, results in precipitation of the active protein, which is collected by centrifugation. It is suspended in 250 ml. of water, and the pH is raised to 8.0 by the addition of dilute ammonium hydroxide. In the presence of the small amount of ammonium sulfate carried down during the centrifugation the protein will not dissolve at any reaction from pH 1 to 10. Solution may be effected by

dialyzing out the salt, but when dialyses are introduced into the fractionation procedure, the total yield is considerably less. The suspension, therefore, is brought to a concentration of 1.25 M ammonium sulfate by the addition of a 2.5 M solution of the salt and the pH is lowered to 5.9 to 6.0. The collected precipitate is subjected to a third treatment of this kind. The final precipitate is suspended in 150 ml. of distilled water and dialyzed in a revolving cellophane bag against running water until very nearly sulfate-free. It is dissolved by the addition of dilute hydrochloric acid to pH 3.5, forming a clear brown solution. This, in one experiment, contained 473 mg. of nitrogen and 49,500 units of parathyroid activity, corresponding to a nitrogen potency of 105 and a yield of 50 per cent of the original activity.

*Adsorption on Benzoic Acid*—A solution fractionated with ammonium sulfate as above, containing 0.6 to 1.0 mg. of nitrogen per ml., is adjusted to pH 3.5. To this, at room temperature, is added very slowly and with efficient stirring one-fourth of its volume of warm 5 per cent sodium benzoate solution, the pH of which had been adjusted to 5.0. No benzoic acid crystallizes from such a solution at 35–40°. During addition of this solution the pH of the mixture is maintained at 3.5, at which precipitation of benzoic acid does not begin until approximately 75 per cent of the benzoate solution has been added. After several hours at 2° or 1 hour in an ice bath with continuous stirring, the benzoic acid is removed by filtration. The mother liquor is warmed to room temperature and treated again with the same quantity of sodium benzoate. Four such adsorptions are usually carried out.

The combined benzoic acid adsorbate, with a little water, is stirred in a beaker with several portions of peroxide-free ether until the solid benzoic acid has been removed. These ether extracts contain considerable amounts of protein material, which may be removed by shaking with a little very dilute acid. This extract is combined with the main aqueous solution, and the remaining benzoic acid is removed by extraction with ether in a separatory funnel. Considerable emulsion formation may frequently be encountered at this stage.

Finally the solution is dialyzed to remove any residual benzoic acid and salt. During dialysis, the excess acid is lost and the pH increases to about 6.0; the hormone is insoluble in this range and precipitates. A clear, brownish yellow solution is formed on the addition of hydrochloric acid to pH 3.5. This solution may be concentrated *in vacuo*, or it may be brought to dryness in the frozen state without appreciable loss in activity. The dry non-hygroscopic material may be kept indefinitely in the cold.

Typical preparations (Lots 164 and 184, respectively) after drying over phosphorus pentoxide at 100° for 2 hours had total nitrogen contents of



12.6 and 13.1 per cent (Kjeldahl procedure), and various preparations had an amino nitrogen content of 6.5 to 7.0 per cent of the total nitrogen (Van Slyke method). In several preparations, negative Molisch tests indicated the absence of carbohydrate. The yield may vary rather widely, but it is frequently near 40 per cent, based on the total activity of the solution first treated with benzoic acid. The nitrogen potencies of several preparations were as follows (the first figure in parentheses indicates the mg. of nitrogen injected): Lot 164, 250 (1.3, Dog 1), 155 (1.3, Dog 2), 310 (1.3, Dog 3), 220 (1.3, Dog 4), 280 (2.4, Dog 3), 140 (2.4, Dog 6); Lot 162, 290 (1.3, Dog 2), 430 (1.6, Dog 3), 300 (1.3, Dog 4), 180 (1.6, Dog 6); Lot 184, 250 (1.5, Dog 1), 410 (1.5, Dog 2), 400 (1.5, Dog 3), 350 (1.5, Dog 4), 100 (1.5, Dog 6) units per mg. of nitrogen.

#### *Other Fractionation Procedures Investigated*

*Isoelectric Precipitation*—It has been recalled that Collip and Clark (1) obtained their most active preparation by a series of precipitations at pH 4.8. The material encountered in this research has been soluble near this acidity, but some purification was accomplished by repeated precipitation at pH 6.0. The products obtained after a series of five to ten such fractionations had a nitrogen potency of 60 to 150 units and represented approximately 20 per cent of the original activity. More satisfactory results were not obtained from precipitations at pH 7.5 or 8.5.

*Charcoal Adsorption*—Adopting the procedure applied by Moloney and Findlay (17) to the purification of insulin, we treated a solution containing 40.3 mg. of parathyroid hormone nitrogen of potency 100 with 4 gm. of Darco. The non-adsorbed fraction (10.4 mg. of N) was inactive, as was that eluted by 12 per cent benzoic acid in 60 per cent ethanol (13.9 mg. of N). A total of 3.4 mg. of inactive nitrogen was further separated by successive extraction with dilute KOH of pH 9.5, 5 per cent acetic acid, and an aqueous alcoholic solution of phthalic acid. The failure to elute the active material led to the use of benzoic acid as adsorbent, from which separation was possible by solution.

*Electrophoresis*—Electrophoresis at 5000 volts in a series of six cells separated by sintered glass discs (18) resulted in precipitation and immobilization of the protein as soon as the pH reached the range of 6 to 10. This, of course, invalidated any attempt at fractionation or determination of the true isoelectric point.

*Electrodialysis*—The activity of a hormone solution maintained at pH 4.0 and electrodialyzed at a potential of 120 volts in the center cell of a three compartment unit separated by cellophane membranes did not undergo any appreciable alteration. It is therefore unlikely that the hormone molecule contains any loosely bound polar group of low molecular weight which is required for physiological activity.

*Acetone and Acetone-Picric Acid Fractionations*—Three otherwise identical parathyroid hormone solutions at pH 2.5, 3.0, and 3.5, respectively, were treated with acetone in varying concentrations. In no case was the difference in nitrogen potency between the insoluble and soluble fractions sufficient to hold promise as a preparative procedure. Fractionation with acetone and picric acid (19) was likewise unsuccessful.

*Trichloroacetic Acid Precipitation*—Concentrations of trichloroacetic acid greater than 1 per cent precipitate active material from partially purified parathyroid extracts; below 1 per cent the hormone tends to remain in solution. There was insufficient difference between nitrogen potencies of the various fractions to warrant further study.

#### DISCUSSION

The opinion has been generally held for 10 years or more (7) that the active principle of the parathyroid gland is either of protein nature or is closely associated with a protein carrier. This is amply supported by the inactivation of the hormone by both acidic and basic hydrolysis (20), and by digestion with proteolytic enzymes (1). Alteration of the functional groups of the protein molecule, such as esterification of the carboxyls (2, 3), also results in inactivation. Activity has always been found to be associated with the protein fractions of glandular extracts; the active material is precipitated by protein reagents and is salted-out of solution under appropriate conditions. Characteristic of the substance is its stability, like that of insulin, to treatment with mineral acid (20) and to rather drastic heating in phenol (2), and also its solubility in aqueous alcohol (1).

Our preparations are undoubtedly of protein nature. This is supported by their general properties, which correspond to those outlined above, and by several additional observations which we have made. The ultraviolet absorption spectrum, for example, is nearly identical with those of numerous other simple proteins reported in the literature (21, 22). It is shown in Fig. 1. There is the typical absorption of the aromatic amino acids, tyrosine, tryptophane, and phenylalanine, but no evidence for the presence of any other absorbing group. The single band has a maximum at  $274\text{ m}\mu$  and an extinction coefficient  $E_{1\text{cm.}}^{1\%} = 10.7$ . These may be compared with values for egg albumin of  $279\text{ m}\mu$  and  $E\ 7.1$ , for horse serum albumin of  $277\text{ m}\mu$  and  $E\ 7.1$ , for a horse serum pseudoglobulin of  $279\text{ m}\mu$  and  $E\ 14.7$ , respectively.<sup>2</sup>

The active principle has also been found to be very sensitive to the action of pepsin. We investigated the possibilities of using this enzyme to effect purification by digestion of accompanying protein impurities, as it has been employed by others in the case of diphtheria antitoxin (Parfentjev

<sup>2</sup> We are indebted to Dr. R. N. Jones for these figures.

(23), Hansen (24), *et al.*). Experiments were carried out at pH 4.1 and 3.6 with relatively little crystalline enzyme, the substrate to enzyme nitrogen ratios being 25 and 100, respectively. In every trial, complete inactivation resulted, indicating that the active principle is itself very readily attacked by pepsin.

There is thus no evidence for the presence of an active group of low molecular weight. The electrodialyses described in the experimental section failed to separate a small polar group from the active material. The ultraviolet absorption spectrum gives no evidence for chromophoric prosthetic groups, and the pepsin digestions indicate an intimate relation between activity and the intact protein molecule.

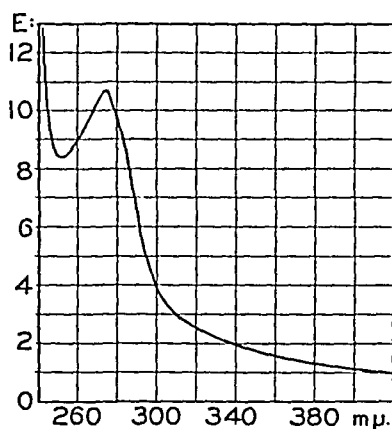


FIG. 1. The ultraviolet absorption spectrum of a parathyroid hormone preparation having a nitrogen potency of 300. The extinction coefficient,  $E$ , is calculated for a 1 per cent solution of protein in a cell 1 cm. long.

All active preparations yet available have unquestionably contained considerable amounts of inert proteins. The so called isoelectric points, variously reported, indicate this. Collip's best preparation precipitated sharply at pH 4.8, being soluble in either more alkaline or more acidic solutions (1). Tweedy and Torigoe (3) referred to the isoelectric point of their material as being at pH 5.8. A preparation described by Allardye (25) precipitated at pH 4.8 when this value was approached from the acid side, or at pH 6.8 when approached from the alkaline side. Our own preparations are soluble in acid solutions up to pH 4.5 or 5.0, then become insoluble on gradual addition of alkali, and remain so until quite high pH values are approached, frequently as high as 10.5 or 11.0. Alkaline suspensions do not dissolve until the pH falls below 5.0. This broad region of insolubility has invalidated any attempt to separate the components

of the extract by electrophoresis and also makes it impossible to study the electrophoretic pattern. It would appear unlikely that there is more than one protein with the property of raising serum calcium, usually attributed to the active principle of the glands; we should therefore be inclined to the belief that the different physical properties of the various preparations are due to variable amounts of protein impurities associated with the active principle.

The ultracentrifugal examination of our most active preparations supports this interpretation. One of these preparations, with a nitrogen potency of 300 units per mg. of nitrogen, was found to consist of 35 per cent of a protein with sedimentation constant equal to  $17S$  and 65 per cent of another protein with a value of  $1.6S$ . In the absence of diffusion constants it is impossible to calculate exact molecular weights for these two components, but the first should have a molecular weight of from 500,000 to 1,000,000 and the second should fall in the group of proteins with molecular weights of 15,000 to 25,000.

In a large scale preparative run with the ultracentrifuge, over 50 per cent of the activity remained in the mother liquor after the complete sedimentation of the heavier component, which appeared as a jelly in the bottom of the tubes. This would indicate that the activity may be associated with the component of lower molecular weight. The fact that a slight loss is always encountered when active preparations are dialyzed against running water also agrees with this interpretation.

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#### SUMMARY

1. A method is described for the preparation of parathyroid extracts of approximately 3 times the activity of any hitherto reported.
2. Present concepts regarding the protein nature of the active principle are reinforced by a study of its pepsin digestion under suboptimal conditions, by its ultraviolet absorption spectrum, and by its stability to electrolysis.
3. Ultracentrifugal study of the preparation shows it to be heterogene-

ous, consisting of at least two components, one of molecular weight of roughly 20,000 and another of 500,000 to 1,000,000.

4. There is some reason to believe that the activity may be associated with the low molecular material.

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# THE KETENE ACETYLATION OF THE PARATHYROID HORMONE\*

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In addition to the numerous reactions of the parathyroid hormone which are typical of proteins, it undergoes several others which indicate a relation between its activity and the presence of free amino (or imino) groups within the molecule. It was observed by Tweedy and Torigoe (1) that its activity was completely destroyed by treatment with formaldehyde, and that the resultant product could be reactivated to the extent of about 25 to 50 per cent by boiling with very dilute hydrochloric acid. The hormone was also completely inactivated by aqueous nitrous acid, a result which might be attributed to reaction with the amino groups. In a further study of this (2) it was found that deamination to the extent of 35 per cent resulted in complete inactivation. This sensitivity to nitrous acid led to the conclusion that some reaction other than deamination was playing a rôle, a belief which was supported by the ease with which the hormone was inactivated by oxidation with hydrogen peroxide. The effect of nitrous acid might therefore be attributed to deamination, oxidation, or simple substitution, resulting in the formation of nitroso derivatives.

The use of ketene, which reacts with free amino groups and the phenolic hydroxyls of tyrosine, offers an admirable means of determining the dependence of the hormone on these respective groupings. Hydrolysis of the O-acetyl residues after complete saturation of the protein with ketene should provide a molecule in which the only alteration would involve acetylation of the free amino groups. The reaction with ketene has accordingly been investigated in much the same manner as that employed by other authors (3-5).

## EXPERIMENTAL

The acetylation of the parathyroid hormone was carried out in the apparatus utilized by Ross and Christensen for treating proteins with carbon suboxide (6). The preparation actually employed was obtained according to the procedure described in the preceding communication (7) and had a nitrogen potency of approximately 180 units. Twice distilled ketene, in 5 times the quantity equivalent to the total reacting groups

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(i.e. the sum of the amino and phenolic hydroxyls) was added over a period of 45 minutes at 0° to a fine suspension of this product containing

TABLE I

*Liberation of O-Acetyl Groups in Acetylated Parathyroid Hormone by Hydrolysis at pH 10.0 and 37°*

Time	Hydrolysis of O-acetyl groups
hrs.	per cent
0	0
0.75	38
1.75	71
4.5	100
8.5	97
44.5	101

TABLE II

*Analytical and Assay Data of Acetylation Experiments*

Experiment	Preparation	Fraction of total amino groups covered	Fraction of total phenolic hydroxyls covered	Nitrogen potency,* u.s.p. units per mg. N
		per cent	per cent	
A	Original protein	0	0	160 (2.2, Dog 3) 200 (2.2, " 4)
	Acetylated (5 equivalents ketene)	42	40	2 (4.8, " 3) 2 (4.8, " 5)
	Acetylated and hydrolyzed	45	4	0 (5.6, " 1) -4 (5.6, " 2) 9 (5.6, " 4) 15 (5.6, " 6)
	Control for hydrolysis	0	0	>150 (<2.7, Dog 2) >140 (<2.7, " 4)
	Original protein	0	0	200 (1.5, Dog 1) 160 (1.5, " 2) 220 (1.5, " 7)
	Acetylated (3 equivalents ketene)	39	19	2 (4.5, " 1) -4 (4.5, " 3) 7 (4.5, " 5) 4 (4.5, " 6)

\* The nitrogen content of the injected dose is indicated in mg. by the first figure in parentheses.

81 mg. of protein nitrogen in 35 ml. of 0.03 M phosphate buffer of pH 6.0. The pH was maintained constant during the acetylation by the addition

of 0.1 N sodium hydroxide. After the reaction was complete, the product was dialyzed against running water for 4 hours to remove much of the inorganic matter present, and finally made to 100 cc., after the pH was adjusted to 4.0, which effected almost complete solution. This solution was analyzed for total nitrogen and for amino nitrogen by the Van Slyke manometric procedure. The semicolloidal nature of the suspensions encountered here, and later, presented no problem in sampling for analysis. The determination of free and acetylated phenolic hydroxyls by the methods of Herriott (8) at pH 8 and 11 gave the per cent of phenolic hydroxyls covered. The protein was kept in solution during the color development by the addition of urea to give a final concentration of 4 M (4).<sup>1</sup> This solution was also assayed for its biological activity (7).

The hydrolysis of O-acetyl linkages within this product was accomplished by digestion for 4.5 hours at 37° in 0.1 M borate buffer of pH 10.0 (see Table I). The bulk of the product dissolved upon acidification to pH 4.0, and the resulting cloudy solution was dialyzed overnight against running water. A complete analysis was carried out as outlined for the acetyl hormone before hydrolysis.

A sample of the original unacetylated hormone was also subjected to the same conditions, pH 10.0 and 37°, for 4.25 hours. This was dialyzed and assayed.

The data obtained from these experiments are assembled in Table II.

#### DISCUSSION

Acetylation with ketene results in complete inactivation of the parathyroid hormone. In the light of present knowledge this must be attributed to alteration of either the free amino groups or the phenolic hydroxyls, or both. It is not felt that the exposure to bodily conditions of pH and temperature during the assay period is sufficiently long to cause an appreciable hydrolysis of O-acetyl linkages. It has been demonstrated that acetic acid bound to phenolic hydroxyls is slowly liberated at pH 7 and 37° (9).<sup>1</sup> Since, however, hydrolysis of oxygen-bound acetyl residues, by which the phenolic groups are restored to the molecule, does not effect reactivation, it must be concluded that the amino groups are an essential part of the biologically active molecule. No statement can be made on the basis of our data regarding the dispensability of the phenolic hydroxyls.

<sup>1</sup> The colorigenic value of the unacetylated hormone is 10 to 15 per cent higher than that obtained when the pH 11 method is applied to the acetylated material. Since presumably all of the O-acetyl groups in the latter are liberated under the conditions of the pH 11 method, additional colorigenic groups of unknown nature have been acetylated and such acetyl linkages are relatively stable at high pH values. No information may be gained from the present data regarding the nature of these groups.



The parathyroid hormone is therefore like diphtheria toxin (10), certain of the pituitary hormones (11, 12), and the gonadotropic hormone of pregnant mare serum (11) in its dependence upon unaltered amino groups, and differs from pepsin (3), insulin (4), and human chorionic gonadotropin (11), which are active regardless of the presence of these groups.

#### SUMMARY

1. Acetylation of the parathyroid hormone with ketene is accompanied by complete biological inactivation.

2. Since liberation of the phenolic hydroxyls by alkaline hydrolysis does not restore activity, it is concluded that the hormone is dependent upon the presence of free amino groups.

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# CARBON SUBOXIDE AND PROTEINS

## VII. MALONYL PEPSIN\*

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For the earlier work on this subject the reader is referred to previous papers (1, 2). The immediate project involves a study of the effect which malonylation has upon the activity and specificity of a typical proteolytic enzyme such as pepsin. Pepsin was chosen because of its availability and the elegant study of its acetylation with ketene, carried out by Herriott and Northrop (3). These authors observed that the activity of pepsin was associated with the presence of free phenolic hydroxyls, since complete acetylation of both amino and phenolic groups inactivated the enzyme, while subsequent liberation of the phenolic hydroxyls by gentle hydrolysis restored the activity. Philpot and Small (4) by the use of nitrous acid also produced evidence to show that unaltered tyrosine residues were essential. The use of the carbon suboxide instead of ketene in this reaction presents the additional factor that a polar carboxyl group, rather than a neutral aliphatic side chain, is introduced into the protein molecule.

### EXPERIMENTAL

Crystalline pepsin, prepared from 1:10,000 Cudahy spongy pepsin by a slightly modified Northrop procedure (5), was treated with carbon suboxide in the usual manner (6). To solutions at pH 5.2 to 5.4 in 0.04 M sodium acetate buffer were added varying amounts of the reagent, as shown in Table I. The products were precipitated twice at pH 3.0 with half saturated magnesium sulfate and were washed with 0.002 N hydrochloric acid. The recovery of protein from such runs varied from 85 per cent in Preparation CP-2a to 46 per cent in Preparation CP-2c.

The procedure of following the reaction by determination of residual amino nitrogen by the Van Slyke method was found to give unsatisfactory results with pepsin, being complicated by the self-hydrolysis of the enzyme to liberate fragments of low molecular weight. Precipitation of the products with hot 5 per cent trichloroacetic acid followed by washing with the same solution, cool, was not effective in removing completely the non-protein nitrogen, nor was it possible to work on a sufficiently large scale to purify the products by crystallization. A satisfactory index of the extent of reaction was obtained by the use of Herriott's method for the determination of free and bound

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phenolic hydroxyls (7), but this, too, was somewhat confused by the presence of products of hydrolysis. It will be recalled that these chromogenic groups when bound in proteins give only 60 per cent of the color of the free amino acids; so that the color value of products containing variable amounts of non-protein N may be difficult to interpret. Of considerable value in estimating the extent of reaction with varying amounts of carbon suboxide has been the curve previously obtained for serum albumin (2).

TABLE I  
*Malonyl Pepsin Preparations*

Preparation*	C <sub>3</sub> O <sub>2</sub> added	Activity [P.u.] <sup>Hb</sup> mg. P. N	Loss of activity	Phenol color pH 8:pH 11	Reaction of phenolic hydroxyls
	<i>equivalents</i>		<i>per cent</i>		<i>per cent</i>
CP-1.....	15	0.040	85	0.37	60
CP-2a.....	1.2	0.13	54	0.70	24
2b.....	4.3	0.030	89	0.42	55
2c.....	8.3	0.014	95	0.23	75
Control, CP-2.....	0	0.28	0	0.92	0
CP-3.....	4.0	0.08	69	0.55	40

\* CP is an abbreviation used to denote "carbon suboxide-treated pepsin" or "malonyl pepsin."

TABLE II  
*Reactivation of Malonyl Pepsin, Preparation CP-3, at pH 4.0*

Conditions of hydrolysis		Activity [P.u.] <sup>Hb</sup> mg. P. N	Fraction of original activity
Time	Temperature		
<i>days</i>	<i>°C.</i>		<i>per cent</i>
0.5	2	0.080	31
7	2	0.086	33
13	25	0.12	45
22	25	0.13	50
36	25	0.15	58

Hydrolysis of the labile O-malonyl group was effected by dissolving 94 mg. of the derived protein in 8 ml. of 0.15 M acetate buffer of pH 4.0 and allowing the solution to stand, first at refrigerator and then at room temperature. The results are shown in Table II. Cold 1.25 N sulfuric acid, frequently employed as a reagent for this purpose, was eliminated by the insolubility of our products in it.

The activity of both the starting material and products was determined by the hemoglobin method of Anson (8) and referred to the total protein

nitrogen, the non-protein N having been found by precipitation of the protein from an aliquot by boiling 5 per cent trichloroacetic acid and by Kjeldahl assay of the filtrate. In this way, our native crystalline pepsin consistently had an activity equal to 0.26. Similar assays of treated products are shown in Tables I and II.

In addition, hydrolyses of casein and of the synthetic substrate carboxybenzoxy-L-glutamyl-L-tyrosine (9) were carried out. The digest of the former contained 30 mg. of casein and 1.0 mg. of enzyme nitrogen per ml. at pH 2.0, and the hydrolysis was followed by the semimicro volumetric Van Slyke procedure. It appears that in earlier enzymic studies with carboxybenzoxyglutamyltyrosine as substrate suspensions were employed (9). It has been found that a perfectly clear solution of pH 4.0 may be obtained by dissolving 40.0 mg. of the peptide in 1.25 ml. of 0.15 M sodium acetate at 40° and diluting with an equal volume of 0.18 M acetic acid. Such solutions were used for our studies. Addition of 0.5 ml. of native pepsin solution containing 3.0 mg. of protein nitrogen at pH 4.0 resulted in 47 per cent hydrolysis of the substrate after 24 hours, as determined in the Van Slyke manometric apparatus.

All digestions were carried out at 40°. Controls were maintained for self-hydrolysis both of enzyme and of substrates. Digests and controls were made up and analyzed in duplicate for the studies with the synthetic substrate.

#### DISCUSSION

The results of the experiments summarized in Table I show quite clearly that malonylation inactivates pepsin. There is a 50 per cent loss in activity when one-fourth of the phenolic hydroxyls has reacted and almost complete inactivation when three-fourths has disappeared. Somewhat higher percentages of the free amino groups may be expected to have reacted at these stages. Gentle hydrolysis of the O-malonyl linkage, as shown in Table II, results in a proportionate return of enzymic activity, indicating the intimate association between the phenolic hydroxyls and activity. This behavior is in every way like that of acetyl pepsin (7).

It should be recalled that the O-malonyl linkage is labile in solution at 40° even at nearly neutral pH values (2); so that the method of enzymic assay must not involve too long exposure to such conditions if conclusions regarding the rôle of tyrosine are to be drawn. The 10 minute digest period of the hemoglobin method is not sufficient to have any effect.

Our main objective has been evidence regarding the influence which malonylation may have upon the *specificity* rather than the over-all *activity* of the enzyme. One line of approach involves the determination of the relative activities of malonyl and native pepsin with several different

substrates. If the specificity of the enzyme were changed by malonylation so that its affinity for one particular kind of group were either greatly enhanced or diminished, then the ratio of activities for the two enzymes would be expected to vary in going from one substrate to another in which different amounts of the appropriate amino acid residues were present. Ideally, such a concept should be tested with an unlimited number of various synthetic polypeptides. Practically, we have had to restrict the study to one polypeptide, carbobenzoxyglutamyltyrosine, which appears to be the best available synthetic substrate for pepsin (9), and to two proteins, casein and serum albumin, chosen on account of their unlike amino acid contents. Unfortunately, the polypeptide mentioned above is not a very satisfactory substrate, as it is relatively insoluble, gives a precipitate of tyrosine on hydrolysis, and is split by pepsin to the extent of only 50 per cent after 24 hours. However, it does offer one means of testing the theory in question. If the activity of pepsin towards this substrate were found to be considerably decreased by malonylation, and that towards one of the proteins were the same or greater afterwards, an alteration of the specificity would be indicated.

Towards this end the experiments of Table III were performed. There is excellent agreement between the ratios of the activities of native and malonyl pepsins when tested with the three substrates. After 20 hours at pH 4.0 and 40° the number of malonic acid residues bound to tyrosine would not be significant (less than 10 per cent of the total tyrosine); so that any effect would have to be attributed to lysine-bound malonyl groups. It may be concluded, therefore, that, not only are the polar  $\epsilon$ -amino groups of lysine unessential for enzymic activity, but also a complete reversal of the charge in these positions has no effect on the specificity of the enzyme.

The availability of various malonyl preparations suggested the study of the digestion of native and malonyl horse serum albumins by native and malonyl pepsins. The results of such experiments are given in Table IV. There is no apparent specific difference in the behavior of native and malonyl pepsins with the two proteins, but there is a decided difference as regards the substrates. The final hydrolysis is about 20 per cent greater with malonyl serum albumin as substrate than with the native protein, regardless of the enzyme involved. In other words, approximately 40 additional linkages within the molecule have been made subject to the action of pepsin by the carbon suboxide treatment. Of the lysine in this preparation 90 per cent has been coupled with malonic acid residues; therefore if a figure of 55 is taken for the total number of lysine residues (2), there are approximately 50 new polar groups of negative charge within the molecule. Oxygen-bound malonic acid need not, of course, be considered. This figure is sufficiently near the number of new linkages split to indicate a relationship between the two.

TABLE III

*Digestions of Various Substrates by N-Malonyl Pepsin*

Enzyme Preparation CP-3 was employed throughout. Experiments with synthetic substrate and casein were begun 12 hours after preparation of the enzyme; those with serum albumin, 3 weeks later, the enzyme cake having been kept under saturated magnesium sulfate in the ice box meanwhile. The serum albumin digest solutions contained 3.2 mg. of substrate N per ml. and 0.32 mg. of enzyme N per ml. in 0.05 M citrate buffer at pH 2.0. Other details are given in the experimental section.

Substrate	Enzyme	pH	Hydrolysis	
			20 hrs.	142 hrs.
			per cent	per cent
Carbobenzoxy-l-glutamyl-l-tyrosine	N-Malonyl pepsin	4.0	36	52
	Native pepsin	4.0	45	60
	Ratio		0.80	0.87
			NH <sub>2</sub> -N per ml.	
Casein (Hammarsten)	N-Malonyl pepsin	2.0	mg. 0.40	mg. 0.52
	Native pepsin	2.0	0.48	0.57
	Ratio		0.83	0.91
Horse serum albumin (10)	N-Malonyl pepsin	2.0	0.150	
	Native pepsin	2.0	0.185	
	Ratio		0.81	

TABLE IV

*Digestion of Native and Malonyl Horse Serum Albumins by Native and Malonyl Pepsins*

The results are expressed as the increase in the ratio of amino to total nitrogen. Conditions in all experiments were such that the ratio of substrate to enzyme nitrogen was 10. In the experiments with native pepsin the substrate concentration was 2.5 mg. of N per ml. and in those with malonyl pepsin, 3.2. Solutions were 0.05 M in citrate buffer of pH 2.0.

Time	Native serum albumin		Malonyl serum albumin	
	Native pepsin	N-Malonyl pepsin, Preparation CP-3	Native pepsin	N-Malonyl pepsin, Preparation CP-3
<i>days</i>				
0.8	0.150	0.157		
2.0	0.175	0.186	0.170	0.202
6*	0.194	0.204	0.214	0.242
7	0.195	0.204	0.232	0.243
11	0.207	0.214	0.252	0.261
18	0.222	0.205	0.266	0.264
25	0.224		0.272	

\* Fresh enzyme was added immediately after these determinations.

The specificity of pepsin towards synthetic substrates (9) is such as to support this idea. Pepsin splits the peptide derivative, carbobenzoxy-l-

glutamyl-*l*-tyrosine more readily than any other substrate, any alteration in either of the two carboxyls or the phenolic hydroxyl depressing considerably the rate of hydrolysis. In accordance with this, carbobenzoxyglutamyl-tyrosinamide is attacked very sluggishly. But, and this is of considerable value to the present discussion, if the tyrosyl carboxyl is bound to glycine instead of ammonia, giving carbobenzoxyglutamyltyrosylglycine, the new carboxyl contributed by glycine, although considerably removed from the peptide linkage which is hydrolyzed, restores the original rate of digestion. Thus the presence of a carboxyl group near but not directly associated with a peptide linkage which is almost labile to pepsin may suffice to effect its ready hydrolysis. It may be in this way that malonylation increases the peptic hydrolysis of serum albumin.

#### SUMMARY

1. Malonylation of the free amino and phenolic hydroxyls of pepsin inactivates the enzyme.
2. Gentle hydrolysis, by which the tyrosyl-bound malonic acid is liberated, results in partial reactivation.
3. The specificity of pepsin is not altered by the presence of carboxyl groups in positions normally occupied by the basic lysyl residues in pepsin. These residues are therefore both unessential for activity and without influence on the specificity of the enzyme.
4. Malonylation of serum albumin increases the number of peptide linkages subject to the action of pepsin.

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## RADIOACTIVE SULFUR STUDIES†

I. SYNTHESIS OF METHIONINE\*.‡ II. CONVERSION OF METHIONINE SULFUR TO TAURINE SULFUR IN DOGS AND RATS. III. DISTRIBUTION OF SULFUR\* IN THE PROTEINS OF ANIMALS FED SULFUR\* OR METHIONINE\*. IV. EXPERIMENTS IN VITRO WITH SULFUR\* AND HYDROGEN SULFIDE\*

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### *I. Synthesis of Methionine\**

Methionine\* has been synthesized (1) by a modification of the method described by Patterson and du Vigneaud (2). The yield was rather low and the method is impracticable when small amounts of the compound are needed at intervals. It has been found that a modification of the Hill and Robson (3) method is more suitable. The synthesis of  $\alpha$ -benzamido- $\gamma$ -chlorobutyric acid ethyl ester is carried out as described by these authors. Then the only *essential* modification is the replacement of the methylmercaptan used by Hill and Robson with benzylmercaptan.

*Method*—Benzylmercaptan\*<sup>1</sup> was prepared as previously described (1). In one experiment 0.53 gm. (4.3 mm) of the mercaptan\* was dissolved in 1 ml. of absolute methyl alcohol containing 0.15 gm. (6.5 mm) of dissolved sodium. After the temperature of the mercaptide solution was raised to its boiling point, 1.4 gm. (5.2 mm) of ethyl  $\alpha$ -benzamido- $\gamma$ -chlorobutyrate<sup>2</sup> were added. The mixture was maintained at the boiling point for 10 minutes to insure completion of the reaction. To the mixture 60 ml. of 0.25 N NaOH were added, and hydrolysis was completed by boiling for 15 minutes. The  $\alpha$ -benzamido- $\gamma$ -benzylthiolbutyric acid\* was not isolated but the hydrolysis was continued by refluxing with 80 ml. of constant boiling point HCl for 5 hours in order to obtain the free S-benzylhomocysteine\*. Benzoic acid was filtered from the cold solution and washed with ice water. After the free HCl was distilled off, the solution was adjusted to pH 5.5 to 6.5, whereupon the S-benzylhomocysteine\* precipitated. It was filtered off, washed with ice water, 95 per cent alcohol, and ether. The yield of

† Aided by grants from the Rockefeller Foundation and Eli Lilly and Company.

‡ The asterisk indicates that the substance contains traces of S<sup>35</sup>.

<sup>1</sup> Most of the sulfur used in these experiments was prepared by neutron bombardment of CCl<sub>4</sub> (4). The S\* was isolated as BaSO<sub>4</sub>\*. The BaSO<sub>4</sub>\* was converted to BaS\* by reduction with hydrogen at 1000° instead of 900° (5).

<sup>2</sup> The melting point of this compound was found to be 62–64° instead of 45° as given by Hill and Robson (3).



the dried S-benzylhomocysteine\* was 0.718 gm. or 75 per cent of the theoretical on the basis of the benzylmercaptan\* used. Without further purification it showed exactly the same behavior in the melting point tube as did recrystallized S-benzylhomocysteine synthesized by the Patterson and du Vigneaud (2) method. Both substances browned at 208° and decomposed at 235–239° (corrected). According to du Vigneaud and Patterson (6) the decomposition point of *dl*-S-benzylhomocysteine is 240–245° (corrected).

The next steps of the procedure are the same as those previously given (1) except that it was found more convenient to methylate the homocysteine by using a solution of methyl iodide in dry ether. The residues from the synthesis may be used to recover sulfur\*.

## *II. Conversion of Methionine Sulfur to Taurine Sulfur in Dogs and Rats*

In a series of papers, Virtue and Doster-Virtue (7, 8) have shown that when cystine, cysteine, cystine disulfoxide, cysteinesulfinic acid, cysteic acid, methionine, methionine sulfoxide, and homocysteine were fed to bile fistula dogs given cholic acid in excess to deplete the taurine and possibly taurine precursors and to provide an ample supply for later conjugation (9) the alcohol-soluble fraction of bile is increased. This is presumptive evidence that the sulfur of these compounds was converted to taurine sulfur. When homocystine was fed, the results were uncertain and, when cystamine was fed, no increase in the alcohol-soluble sulfur-containing fraction of bile was observed. Virtue and Doster-Virtue (8) state, "No proof is yet available that methionine is actually changed to taurine."

The present experiments were undertaken to show whether or not methionine sulfur can be converted to taurine sulfur by the dog and rat. Previous studies (10, 1) have proved that methionine sulfur can be converted to cystine sulfur by the rat. On the basis of extra oxygen consumption it has been concluded (11) that tissue slices and crude enzyme preparations can oxidize cysteine to cysteic acid.

### *Experiments with Bile Fistula Dogs*

*Dog 1 (Orientation Experiment)*—Beginning 2 days after the operation and each day thereafter, 2.4 gm. of cholic acid were administered to the dog. 10 mg. of methionine\* were fed on the 6th day and the bile was collected daily for 4 days. Protein was precipitated from the bile with alcohol. The residues from aliquots of these alcoholic solutions were digested with the Pirie reagent (12). The sulfate content of the dissolved residue was determined by precipitating benzidine sulfate and titrating with standard alkali, with phenol red as indicator (13). After the titration most of the benzidine was filtered from the cold solution, the filter was

washed with water, and sufficient extra  $\text{H}_2\text{SO}_4$  (carrier sulfate) was added to give a total of 1 milliequivalent of sulfate. All the sulfate was precipitated from the solution as  $\text{BaSO}_4$ . Samples<sup>3</sup> for the determination of radioactivity were prepared (1). The radioactivity was determined by use of a screen-walled counter tube (14) and Geiger-Müller counter.<sup>4</sup> Unless otherwise stated, all other products were similarly treated in order to determine both the sulfur content and the radioactivity.

Taurine was isolated from the alcohol-soluble fraction corresponding to 143 ml. of the 1st day's bile by the following method. The residue from the alcoholic solution was hydrolyzed by heating on the steam bath for 8 hours with 50 ml. of 2.5  $\text{N}$   $\text{NaOH}$ . The solution was taken down to dryness, any residual 95 per cent alcohol-soluble material was extracted, and the taurine was removed from the salt residue by extracting three times with 5 ml. portions of 12  $\text{N}$   $\text{HCl}$ . The  $\text{HCl}$  was evaporated and the taurine-containing residue was taken up in a few ml. of water. The product crystallized from the filtered solution during the addition of 4 volumes of 95 per cent alcohol. It was recrystallized three times from water-alcohol. The pure white product had the same crystalline form as a known specimen of taurine crystallized in a similar manner (15). Samples for counting (determination of radioactivity) were prepared from the taurine. The residues and mother liquors from the taurine isolation were similarly digested and converted into a sample. The radioactivities of the samples are given in Table I.

*Dog 2*—The treatment of Dog 2 was similar to that of Dog 1 except where it is otherwise indicated. 1 gm. of cholic acid, first given 2 days after the operation, was administered morning and evening until the 7th day. On this day the dog (weight 14.3 kilos) was given 53.1 mg. of methionine\* and 1.5 gm. of cholic acid by stomach tube. An additional gm. of cholic acid was fed 6 hours later. Two 12 hour collections of bile were made during the ensuing period. The animal was then sacrificed. Taurine was isolated from the first and second 12 hour specimens of bile treated separately by an elaborated version of that used in the preceding experiment. The crude taurine preparations were crystallized once from water-alcohol. Small residues obtained when these products were dissolved in 12  $\text{N}$   $\text{HCl}$  were discarded, the  $\text{HCl}$  was evaporated, and the taurine recrystallized as before. Two recrystallizations were then made from water and one from water-alcohol. The final products had the same crystalline form as the previous specimen. A known specimen of taurine similarly recrystallized

\* The term *sample* is used to designate the  $\text{BaSO}_4$ \* used for the estimation of the radioactivity.

<sup>4</sup> The Geiger-Müller counter was of the type manufactured by the Cyclotron Specialties Company, Moraga, California.

decomposed at 328° (corrected) and the isolated taurine specimens at 329° (corrected). White and Fishman (16) give a decomposition point of 328°  $\pm$  1° (corrected).

TABLE I  
*Radioactivities of Constituents in Bile of Bile Fistula Dogs Fed Methionine\**

Dog No.	Bile constituent	Radio-activity	Specific activity†	Per cent replacement‡
		<i>counts per min.</i>	<i>counts per min.</i>	
1§	Alcohol-soluble fraction of bile, 1st day	174 $\pm$ 27		
	"      "      "      2nd "	109 $\pm$ 15	164 $\pm$ 22	0.029
	"      "      "      4th "	134 $\pm$ 16	124 $\pm$ 15	0.022
	Taurine (bile, 1st day)	111 $\pm$ 15	207 $\pm$ 28	0.036
	Residues ( " 1st " )	87 $\pm$ 14	175 $\pm$ 28	0.031
2	$\beta$ -Naphthalenesulfotaurine (bile, 1st 12 hrs.)¶	290 $\pm$ 32	450 $\pm$ 50	0.21
	"      ( " " 12 " )	301 $\pm$ 20	482 $\pm$ 30	0.22
	Taurine (bile, second 12 hrs.)	442 $\pm$ 20	344 $\pm$ 16	0.16
	"      ( " " 12 " )	424 $\pm$ 20	340 $\pm$ 16	0.16
3**	Alcohol-soluble fraction of bile (6-18 hrs.; 13 ml.)	254 $\pm$ 24		
	Alcohol-soluble fraction of bile (18-24 hrs.; 8 ml.)	114 $\pm$ 30		
	Taurine (bile 6-18 hrs.)	284 $\pm$ 24		
	"      ( " 6-18 " )	278 $\pm$ 24		
	Organic S in protein fraction of bile (total)	88 $\pm$ 20		
	Inorganic " " " " " " "	-4 $\pm$ 14		
	Alcohol-soluble sulfate (bile, 0-6 hrs.)	-4 $\pm$ 14		
	"      "      ( " 6-18 " )	-6 $\pm$ 14		
	"      "      ( " 18-24 " )	-5 $\pm$ 14		

† Specific activity = radioactivity per millicivalent of sulfur.

‡ Per cent replacement =  $\frac{\text{specific activity of sample}}{\text{specific activity of methionine}} \times 100$ .

§ 10 mg. of methionine\* fed (77,000  $\pm$  4700 counts per minute); specific activity, 570,000  $\pm$  35,000 counts per minute.

|| 53.1 mg. of methionine fed (156,200  $\pm$  5400 counts per minute); specific activity, 219,000  $\pm$  7600 counts per minute.

¶ In order to obtain the specific activity of the taurine sulfur in this derivative, the specific activity as defined has been multiplied by 2.

\*\* 103.5 mg. of methionine fed (162,000  $\pm$  17,000 counts per minute); specific activity 117,000  $\pm$  12,000 counts per minute.

The taurine from the 1st 12 hours bile was converted to the sodium salt of  $\beta$ -naphthalenesulfotaurine by the technique given by Schmidt and Cerecedo (17). The sodium salt was recrystallized from 50 per cent alcohol. The dried product had the same crystalline structure as a known specimen.

Both preparations decomposed at  $245^{\circ}$  (corrected). Bergell (18) gives  $247^{\circ}$  (corrected).

The radioactivity data obtained on the  $\text{BaSO}_4$  precipitates made from the digestions of the taurine and its derivative are given in Table I.

*Dog 3*—This dog was fed 1 gm. of sodium cholate daily for 6 days, starting from the 1st day after the operation. On the 8th day, 1 gm. of cholic acid was fed morning and evening. On the following day 103.5 mg. of methionine\* and 1 gm. of cholic acid dissolved in 100 ml. of 20 per cent glucose were given. At this time the dog weighed 10.4 kilos. During the following 24 hours bile was collected in periods of 6, 12, and 6 hours. The animal was then sacrificed.

Taurine was isolated from 48 ml. of the 6 to 18 hour bile by the preceding methods. The decomposition point of this taurine was compared with that of the known specimen by placing the melting point tube in a bath at  $324^{\circ}$  and then raising the temperature to the point of decomposition. The decomposition points were as follows: taurine from bile  $331^{\circ}$  (corrected), known specimen  $332^{\circ}$  (corrected). The temperatures are slightly higher than those observed previously.

To the alcohol-soluble fractions of the first and third collections of bile and to 13 ml. of the second collection, all dissolved in water, carrier sulfate was added and  $\text{BaSO}_4$  was precipitated. After the  $\text{BaSO}_4$  was filtered off, the residue was digested to convert the organic sulfur in the filtrate to sulfate. This was likewise precipitated. The protein, filtered from the alcoholic solutions of the three bile specimens, was washed with alcohol and then with 2 per cent trichloroacetic acid and hydrolyzed for 24 hours with 8 N HCl. From the hydrolysate, after removal of most of the HCl and addition of carrier sulfate,  $\text{BaSO}_4$  was precipitated. The amino acid sulfur in the filtrate was also converted to sulfate. The radioactivities of these various  $\text{BaSO}_4$  precipitates and of the  $\text{BaSO}_4$  obtained from the crystalline taurine are given in Table I.

*Results*—The data collected in Table I show quite definitely that all of the isolated specimens of taurine contained significant amounts of radioactive sulfur. This cannot be attributed to traces of a sulfur-containing contaminant with a very high specific activity because the specific activity of the isolated taurine was always higher than that of the residues. Moreover, the methods used were such as to remove any probable contaminant. Therefore, the results show that in the dog methionine sulfur can be converted to taurine sulfur.

#### *Experiments with Bile Fistula Rats*

Bile fistulas were made on adult rats weighing between 260 and 300 gm. by the method of Sawyer and Lepkovsky (19). Bile was collected from

the bulbs three times a day. The rats were fasted for 1 day before the operation and the fast was continued after the operation.

The sulfur in the alcohol-soluble fraction of the bile was determined either by digestion with the Pirie reagent or with an alkaline Denis reagent (20). In the first case the sulfate was precipitated with benzidine and determined by titration; in the second by weighing as  $\text{BaSO}_4$ . The rat bile contained 0.027 to 0.040 per cent sulfur in the alcohol-soluble fraction.

Amino nitrogen was determined on the alcohol-soluble fraction both before and after hydrolysis with sodium hydroxide according to the method of Schmidt and Dart (21). When the determination of amino nitrogen was carried out on the equivalent of 0.4 ml. of bile, the values obtained from the hydrolyzed and unhydrolyzed bile were small. In most cases, therefore, determinations were made on larger aliquots of the alcohol-soluble fraction. The data are given in Table II.

TABLE II  
*Amino Nitrogen in Alcohol-Soluble Fraction of Rat Bile*

Rat No.	Free	Bound	Rat No.	Free	Bound
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>
1	0.027	0.022	5	0.009	0.015
2	0.024	0.000	6	0.009	0.014
3	0.012	0.008	7	0.013	0.012
4	0.030	0.023	8	0.012	0.014

Methionine\* was fed to six bile fistula rats and the radioactivity in the alcohol-soluble fraction of the bile was determined. The data, collected in Table III, show that a radioactive sulfur compound other than methionine or inorganic sulfate and possibly taurine was present in this bile fraction. The sulfur of this compound apparently comes from that of methionine. It should be noted that the dogs were fed cholic acid, whereas the rats received none of this substance.

### *III. Distribution of Sulfur\* in the Proteins of Animals Fed S\* or Methionine\**

#### *Fate of Elementary Sulfur\* Fed to Normal Rats*

It has been shown that the rat cannot utilize elementary sulfur in lieu of cystine or methionine (22). When fed, most of the sulfur appears in the urine as sulfates (23). The possibility remains that small amounts of sulfur are synthesized into utilizable organic form but that the rate of synthesis is insufficient to supply the requirements of the organism. An attempt has been made to detect such a synthesis by feeding colloidal

sulfur\* to rats. The animals were sacrificed either 48 hours after a single dose or on the 9th day following eight daily doses. A search was made for radioactivity in the proteins of the internal organs.

*Method*—Three normal adult rats on the stock diet were fed 1.00 ml. of a colloidal solution of sulfur\* (10 per cent gelatin containing 1 mg. of sulfur per ml.) by stomach tube. 48 hours later the rats (Nos. 1, 2, 3) were sacrificed. Three more rats (Nos. 4, 5, 6) were fed the same dose of sulfur each day for 8 days and were sacrificed on the 9th day. Protein preparations from the internal organs of all these rats were made as follows: The well minced tissue was precipitated with 8 per cent trichloroacetic acid, washed well with the same reagent, and then extracted with alcohol

TABLE III  
*Experiments on Bile Fistula Rats*

Rat No.	Days after operation†	Methionine* fed	Period of bile collection	Radioactivity of methionine* fed	Radioactivity of alcohol-soluble fraction of bile
		mg.	hrs.	counts per min	counts per min.
1	3	1.0	40	1600	105‡
2	3	1.0	40	1600	39§
3	1	4.4	96	1730	62
4	2	4.4	72	1730	25
8	2	8.8	30	3460	180‡
10	2	4.4	30	1730	43

† All animals were maintained in the fasting condition.

‡ 0.5 mm of sodium sulfate was added to the bile before precipitation with alcohol.

§ The bile was hydrolyzed with NaOH, 0.4 mm of taurine was then added, the total taurine was isolated, and the compound recrystallized three times.

|| 1.4 mm of sodium sulfate and 1.3 mm of methionine were added to the bile before addition of alcohol.

and ether. The products were dried in the oven at 100° and afterwards over KOH in the vacuum desiccator. In the case of Rats 1, 2, and 3, samples were made from liver protein and from the combined proteins of the other internal organs but in the case of Rats 4, 5, and 6, all the internal organs were taken together. The skin and muscular tissue were in all cases discarded after trichloroacetic acid extracts were made. Special care was taken to wash out the alimentary canal.

The urine and feces of Rats 1, 2, and 3 were collected separately. The urines were hydrolyzed by the usual method to convert the ethereal sulfate to free sulfate. Radioactivity determinations were made on the total sulfur and sulfate sulfur in aliquots of the hydrolysate.

The feces of each rat were extracted with 40 ml. of hot 95 per cent alcohol

half saturated with sulfur and then with two 20 ml. portions of hot alcohol. The alcohol was evaporated from the combined extracts, and the sulfur-containing residues were oxidized to sulfate, which in turn was converted into  $\text{BaSO}_4$  samples. The feces residue was extracted twice with 5 per cent trichloroacetic acid. After oxidation and addition of sufficient carrier sulfate,  $\text{BaSO}_4$  samples were prepared.

*Results*—It is quite obvious from the results shown in Table IV that no detectable amount of sulfur\* was introduced into the proteins. A count of 20 per minute or more in a sample would have been positively detected; so that in the case of Rats 1, 2, and 3 less than 0.3 per cent of the sulfur fed

TABLE IV  
*Fate of Sulfur\* Fed to Normal Rats*

Substance	Rat 1	Rat 2	Rat 3
	<i>counts per min.</i>	<i>counts per min.</i>	<i>counts per min.</i>
Proteins from liver.....	$-2 \pm 10$	$-2 \pm 10$	$-6 \pm 10$
"    "    internal organs.....	$-8 \pm 10$	$0 \pm 10$	$6 \pm 10$
Total urinary sulfates†.....	$4500 \pm 400$	$4600 \pm 300$	$4780 \pm 300$
"    "    S†.....	$4610 \pm 300$	$4360 \pm 300$	$4160 \pm 600$
Feces sulfates†.....	$145 \pm 26$	$112 \pm 20$	$308 \pm 52$
"    S†.....	$186 \pm 32$	$14 \pm 12$	$82 \pm 14$
	Rat 4	Rat 5	Rat 6
Proteins from internal organs†.....	$10 \pm 10$	$2 \pm 10$	$-4 \pm 10$

Rats 1, 2, and 3 were fed 1 mg. of sulfur\* ( $7200 \pm 330$  counts per minute) as a single dose. Rats 4, 5, and 6 were fed 8 mg. of sulfur\* ( $57,600 \pm 2640$  counts per minute) in 1 mg. doses per day for 8 days.

† Estimations made on 48 hour collections.

‡ Proteins from internal organs include those of the liver.

could have been present in any one of the tissue protein samples, and in Rats 4, 5, and 6, less than 0.035 per cent.

Table IV also shows that a considerable part of the radioactivity was not accounted for. It is difficult to explain this because very little was detectable in the trichloroacetic acid extracts of the skin and muscle.

#### *Introduction of Sulfur\* into Proteins of Bile Fistula Rats and Dogs Fed Methionine\**

The results of feeding methionine\* to bile fistula rats and dogs showed that only a small fraction of the methionine sulfur\* appeared in the bile, and it was soon found that only a part of the balance was present in the urine and feces. Consequently, the sulfur in the tissue proteins was examined for radioactivity.

*Method*—The tissues were removed from the animals (Rats 1 and 2 and Dogs 2 and 3) as soon as possible after sacrifice and protein preparations were made as before except that 4 per cent trichloroacetic acid was used instead of 8 per cent. The total protein sulfur was converted to sulfate with the Pirie reagent and the amount of sulfate in the digests was determined by titration of the precipitated benzidine sulfate. In most cases aliquots of the filtrate from the benzidine sulfate titration containing 0.5 mm of sulfate were taken for the preparation of samples for radioactivity determination. Determinations were made on duplicate samples except when the organs were too small to allow this to be done (*e.g.*, thyroid). In the case of Dog 3, many of the samples were checked by determining the radioactivity in two different counter tubes.

*Results*—The combined data from Rats 1 and 2 in Table V show that 56 per cent of the methionine\* was introduced into the rat proteins and only 36 per cent was oxidized to sulfate (this includes sulfate in the tissues).

TABLE V

*Distribution of Sulfur after Feeding Methionine\* to Bile Fistula Rats (Nos. 1 and 2)†*

Substance	Per cent of dose administered	Substance	Per cent of dose administered
Total sulfate.....	36	Lungs.....	2
Trichloroacetic acid-soluble S.....	4	Carcass.....	19
Liver.....	14	Skin and hair.....	2
Kidneys.....	5	Bile protein.....	2
Gastrointestinal tract.....	9	“ (alcohol-soluble fraction).....	5
Genitourinary tract.....	3		

† See Table III.

Attention should be drawn to the fact that these rats were fasting and that the dose of methionine\* was small; namely, 1 mg. per rat.

In Table VI are shown the results obtained from Dog 2 which was fed 3.7 mg. of methionine\* per kilo after fasting 6 days and of Dog 3 which was fed 10 mg. of methionine\* per kilo after fasting 8 days. It is evident from the results that the specific activities in the total sulfur fraction of the proteins from the different tissues are very variable. The very high specific activity in the intestinal mucosa and the very low specific activities in the muscular tissues are especially noteworthy. It is possible that the relatively high activity observed in intestinal muscle may be due, in part, to contamination with mucosa.

#### *Experiments with Proteins\* of the Dog*

In the case of Dog 3, the liver protein was fractionated by the method of Banga and Szent-Györgyi (24) in order to ascertain whether or not the



specific activities of the sulfur in the albumin-globulin fraction were the same as in the so called structural protein 1. Also the total liver protein was hydrolyzed, cystine was isolated, and the specific activity of the cystine sulfur was compared with that of the sulfur remaining in solution (methionine sulfur\*).

TABLE VI

*Specific Activities and Per Cent Replacement of Sulfur in Proteins Isolated from Tissues of Bile Fistula Dogs Fed Methionine\**

Protein	Dog 2		Dog 3	
	Specific activity	Re- place- ment	Specific activity	Re- place- ment
	counts per min.	per cent	counts per min.	per cent
Liver.....	342 $\pm$ 16	0.16	475 $\pm$ 10	0.41
Kidney.....	208 $\pm$ 17	0.10	352 $\pm$ 20	0.30
Spleen.....	85 $\pm$ 11	0.04	208 $\pm$ 9	0.18
Lungs.....	138 $\pm$ 13	0.06	197 $\pm$ 12	0.17
Brain.....			93 $\pm$ 12	0.08
Submaxillary gland.....			287 $\pm$ 22	0.25
Thyroid gland.....			178 $\pm$ 70	0.15
Pancreas.....	283 $\pm$ 38	0.13	547 $\pm$ 23	0.47
Leg muscles.....	20 $\pm$ 17	0.01	12 $\pm$ 6	0.01
Ventricular muscle.....			78 $\pm$ 14	0.07
Diaphragm.....	38 $\pm$ 10	0.02	110 $\pm$ 12	0.09
Red blood corpuscles.....			11 $\pm$ 5	0.01
Fibrin.....	337 $\pm$ 40	0.15		
Pseudoglobulin.....	298 $\pm$ 90	0.14		
Albumin.....	340 $\pm$ 16	0.16		
Stomach muscle.....	71 $\pm$ 24	0.03	116 $\pm$ 30	0.10
Small intestinal muscle.....	137 $\pm$ 24	0.06	272 $\pm$ 8	0.23
Large " ".....	68 $\pm$ 14	0.03		
Stomach mucosa.....			281 $\pm$ 22	0.24
Intestinal ".....			943 $\pm$ 22	0.81
Duodenal ".....	380 $\pm$ 10	0.17		
" submucosa.....	366 $\pm$ 39	0.17		

See Table I for dosages and definition of terms.

The plasma proteins were separated by ammonium sulfate precipitation (25). Experiments of a preliminary nature were also carried out in an attempt to determine whether or not plasma proteins constitute a multi-component system in dynamic equilibrium.

*Fractionation of Liver Protein*—The fresh minced liver was extracted at 0° with 3 volumes of a solution which had the following constituents in the concentrations indicated: 5 M urea, 0.6 M KCl, 0.01 M Na<sub>2</sub>CO<sub>3</sub>, and 0.04 M NaHCO<sub>3</sub>. Practically all the liver dissolved to give an

opalescent solution. The extract was then divided into two equal portions and 5 volumes of water were added to each. The first portion (a) was adjusted to pH 6 with acetic acid and the second portion (b) to pH 7. The protein precipitates were separated by centrifuging, washed with ice water, and then with 4 per cent trichloroacetic acid, alcohol, and ether, and made into dry protein samples. The centrifugate (b) was adjusted to pH 6, which resulted in the appearance of more precipitate. This precipitate was treated like the two previously obtained. (When additional acetic acid was added to the filtrates at pH 6, only a cloudiness appeared.) Residual soluble protein (albumin and globulin) in these solutions was precipitated by addition of trichloroacetic acid to a concentration of 4 per cent. Samples for counting were prepared from these protein fractions.

Part of the total liver protein preparation was hydrolyzed by refluxing for 24 hours with 8 N HCl. Cystine was precipitated from the hydrolysate as the cuprous mercaptide (26). It was well washed with buffer of pH 3.5. The sulfur of both the precipitated cystine and of the methionine remaining in solution was oxidized to sulfate and the amount estimated. Its radioactivity was determined.

*Experiments with Plasma Proteins*—The proteins were separated from defibrinated plasma by ammonium sulfate fractionation. Euglobulin\* was precipitated by 33 per cent, pseudoglobulin\* by 46 per cent, Albumin 1\* by 64 per cent, and Albumin 2\* by complete saturation with ammonium sulfate. The subsequent treatment was as follows:

(a) The pseudoglobulin\* precipitate was dissolved in water and dialyzed free from sulfate. It was then dialyzed against a large volume of a solution, 0.14 M with respect to NaCl and 0.0156 M with respect to a phosphate buffer of pH 7.2. Finally most of the phosphate was removed and the NaCl concentration raised to 0.154 M. A little of the protein did not go into solution. It was filtered off. A mixture of 24 ml. of the pseudoglobulin solution and 38 ml. of ordinary defibrinated dog plasma was prepared. After the mixture had stood 12 hours at room temperature, the proteins were separated. However, the pseudoglobulin was taken out at between 30 and 49 per cent saturation with ammonium sulfate instead of between 33 and 46 per cent as previously. The separated proteins were washed with trichloroacetic acid as usual and samples for the determination of radioactivity were prepared.

(b) The combined Albumin 1\* and 2\* precipitates were dialyzed free from sulfate and, after the solution was filtered, an experiment similar to that given under (a) was carried out. 50 ml. of the albumin solution were mixed with 75 ml. of defibrinated dog plasma. A few drops of toluene were added and the mixture was incubated at 37°. The proteins were separated from half of the mixture after 24 hours and from the other half after 72

hours. The albumin was separated between 44 per cent and complete saturation with ammonium sulfate. Samples for the estimation of radioactivity were prepared from the precipitated proteins.

*Results*—The protein precipitated from portion (a) at pH 6 comprised 79 per cent of the total soluble liver protein and had a specific activity of  $484 \pm 18$  counts per minute, that precipitated at pH 7 from portion (b) comprised 47 per cent of the total and had an activity of  $513 \pm 25$  counts per minute, and the protein precipitated by adjusting this solution to pH 6 comprised 29 per cent of the total and had a specific activity of  $491 \pm 30$  counts per minute. The soluble protein comprised 22 to 24 per cent of the total. It had an activity of  $469 \pm 23$  counts per minute. From these results it appears that the specific activities of the various protein fractions were the same within the error of measurement. However, as might be expected, this was not the case with the specific activities of the isolated cystine and that of the residual (methionine) sulfur. The specific activity of the cystine was  $411 \pm 14$  and that of the methionine  $633 \pm 31$ . It is apparent from this result that the conversion of methionine sulfur to cystine sulfur is not a slow process. The same conversion was also shown in the case of the bile fistula rats, Nos. 3, 4, 8, and 10. Radioactive cystine was found in the liver and kidney proteins of all of these animals.

The results of the *in vitro* experiments with radioactive protein fractions added to ordinary defibrinated dog plasma are given in Table VII. It is shown that there is no considerable transfer of radioactivity from either pseudoglobulin or albumin to the other proteins in the plasma. The proteins added to the plasma did not dissociate into components common to the other plasma proteins; they retained their individualities. However, this may not be strictly true, particularly with regard to euglobulin and pseudoglobulin. The data do not exclude the possibility of some exchange between these two proteins.

#### *IV. Experiments in Vitro with Sulfur\* and Hydrogen Sulfide\**

##### *Oxidation of Cysteine with Sulfur\**

Guthrie and Allerton (27) have reinvestigated the reaction which leads to the formation of  $\text{H}_2\text{S}$  when thiol compounds such as cysteine, glutathione, and proteins are treated with sulfur. The type reaction is  $2\text{RSH} + \text{S} \rightarrow \text{RSSR} + \text{H}_2\text{S}$ . There is no proof that the sulfur in the  $\text{H}_2\text{S}$  formed in this reaction originates from the elementary sulfur used. Hence this possibility was investigated by using sulfur\* in the reaction.

*Method and Results*—An alcoholic solution of sulfur\* (12 mg. per cent) was prepared. With an excess of cysteine in a phosphate buffer of pH 6.7 the sulfur\* in part of this solution was reduced. The  $\text{H}_2\text{S}$  was collected and oxidized to sulfate with alkaline  $\text{H}_2\text{O}_2$  and the cystine-cysteine re-

covered from the solution. Samples for radioactivity determination were prepared.

The radioactivity of the  $H_2S$  evolved accounted for most of that added and none was found in the cystine. The radioactivity of the sulfur used, in counts per minute, was  $4250 \pm 500$ ; that of the cystine-cysteine recovered was  $8 \pm 15$ . This experiment and others done under slightly

TABLE VII

*Equilibration of Serum Pseudoglobulin\* and of Serum Albumin\* with Proteins of Normal Defibrinated Dog Plasma*

Protein fractions	Incubation period		
	Pseudoglobulin*, 12 hrs. at 20°	Albumin*, 24 hrs. at 37°	Albumin*, 72 hrs. at 37°
	counts per min.	counts per min.	counts per min.
Original pseudoglobulin*.....	328 $\pm$ 19		
Pseudoglobulin from mixture.....	235 $\pm$ 17	-6 $\pm$ 7 }	6 $\pm$ 7
Euglobulin from mixture.....	34 $\pm$ 9	-6 $\pm$ 7 }	
Original Albumin* 1 and 2.....		183 $\pm$ 15	183 $\pm$ 15
Albumin 1 from mixture.....	17 $\pm$ 7 }	151 $\pm$ 14	172 $\pm$ 15
" 2 " " .....	6 $\pm$ 7 }		

The ratio of pseudoglobulin\* added to the pseudoglobulin in the plasma was 2. The ratio between the albumins was 0.3.

different conditions show that less than 0.5 per cent of the sulfur\* entered the cystine.

#### *Decomposition of Cystine (and Cysteine) in Alkali in Presence of $H_2S^*$*

The decomposition of cystine, cysteine, and similar compounds in alkali has been studied by many workers; that of cystine is complex (28) and many products have been detected. The primary reaction is evidently the splitting of  $H_2S$  from the cystine (see also (29)). If this reaction is reversible, cystine (or cysteine) might be continually reformed but the reversal of the reaction would be difficult to detect because of secondary irreversible decompositions. Therefore, an attempt was made to answer this question by the use of  $H_2S^*$ .

*Method and Results*—100 mg. of cystine were dissolved in 50 ml. of a solution 1 N in NaOH and 0.051 N in  $H_2S^*$  and heated for 3 hours in a closed vessel at 100°. After the  $H_2S$  was removed from the solution and the pH adjusted to 5, the undecomposed cystine was precipitated. This cystine was reprecipitated from an acid solution, washed with water and alcohol, and converted into a  $BaSO_4$  sample. The cystine remaining in the filtrates was precipitated as the cuprous mercaptide. Part of the mercaptide was converted directly into a  $BaSO_4$  sample. The other part was decomposed

with  $\text{H}_2\text{S}$ , the copper sulfide was filtered off, and the cystine remaining in solution was precipitated by adding *l*-cystine as ballast and adjusting the pH. This cystine was reprecipitated from acid solution and washed.

The  $\text{H}_2\text{S}$  used had a radioactivity of  $580,000 \pm 58,000$  counts per minute; the main cystine precipitate,  $800 \pm 200$ ; the whole mercaptide precipitate,  $1280 \pm 40$ ; the whole cystine present in the mercaptide filtrate,  $0 \pm 40$ . The fact that the mercaptide was radioactive but that the cystine isolated by means of added ballast was inactive shows that  $\text{H}_2\text{S}$  (?) was not completely removed from the solution from which the mercaptide and also the original cystine were precipitated. Therefore, it is probable that the  $800 \pm 200$  counts per minute found in the original cystine were due to contamination. Other similar experiments support this conclusion.

In another experiment cystine was replaced by cysteine, with conditions otherwise very similar to the preceding. The purified cystine had an activity of  $0 \pm 10$  counts per minute, whereas the original  $\text{H}_2\text{S}$  had about the same activity as the  $\text{H}_2\text{S}^*$  used in the above experiment. Therefore, the reaction leading to the splitting of  $\text{H}_2\text{S}$  from cystine and cysteine is not appreciably reversible under the conditions of these experiments.

#### DISCUSSION

For purposes of this discussion it will be assumed that the introduction of radioactivity into proteins after methionine feeding represents a new synthesis of peptide bonds rather than exchange of sulfur between the free sulfur\*-containing amino acids and those combined in proteins. It is assumed further that the synthesis of such bonds in bile fistula animals proceeds at approximately the same rate as in the normal animal.

The results in Tables V and VI show that in many tissues the rate of turnover of the sulfur-containing amino acids must be high and in this respect the results are in accord with those obtained by Schoenheimer and his coworkers (30). However, it should be noted that the animals used in this work were fasted and single doses of methionine\* were given, whereas the other workers fed their marked amino acids in larger amounts over a period of several days as a supplement to the diet.

Schoenheimer and coworkers have concluded that no distinction can be made between endogenous and exogenous metabolism. The results reported here support this conclusion. Recently Burroughs, Burroughs, and Mitchell (31) have made the following statement, "In all likelihood, the chemical reactions that Schoenheimer has detected by means of isotopic nitrogen between dietary amino acids and tissue proteins relate not to the fixed proteins of the cells, indispensable to their normal functioning, but to the dispensable reserve proteins, readily subject to mobilization by many experimental procedures and as readily reformed." As previously indicated, the animals used in these experiments were fasted (Rats 1 and

2, 3 days; Dog 2, 6 days; Dog 3, 8 days) before methionine\* was fed; consequently their reserve proteins must have been largely depleted. Nevertheless considerable methionine\* was introduced into the tissue proteins. Consequently the supposition of Burroughs and coworkers finds no support in the experimental results reported. It is indicated that the total cellular protein is labile.

We are indebted to Miss Lura M. Morse for preparing the bile fistula rats and estimating the amino nitrogen, to Dr. L. M. Montgomery and Dr. C. E. Nelson for preparing the bile fistula dogs, and to Mr. Ellery C. Stowell, Jr., for making some of the radioactivity determinations. The radioactive sulfur was kindly supplied by Dr. E. O. Lawrence and Dr. M. D. Kamen of the Radiation Laboratory of this University.

#### SUMMARY

1. A convenient method for the synthesis of methionine\* (containing trace amounts of radioactive sulfur,  $S^{35}$ ) is described.

2. Taurine\* was isolated from the bile of dogs fed methionine\*. In the dog (and probably in the rat) the sulfur of methionine is used in part for the synthesis of taurine.

3. By feeding sulfur\* to rats it was shown that less than 0.035 per cent of the sulfur\* fed was contained in the proteins of their internal organs. Therefore, it is improbable that the rat can utilize elementary sulfur for the synthesis of the sulfur-containing amino acids.

4. A large part of the sulfur\* (as methionine and cystine) is contained in the proteins of fasted bile fistula dogs and rats fed methionine\*. The per cent replacement of the sulfur in proteins with sulfur\* varies in different tissues, but the different protein fractions of the liver and plasma show the same per cent replacement.

5. The evidence indicates that serum albumin is not a system of components that are common to the other serum proteins. The situation with regard to pseudoglobulin and euglobulin is not clear.

6. When cysteine is oxidized with sulfur\*, no sulfur\* is found in the cystine formed.

7. When cystine (or cysteine) is partly decomposed in alkaline solution in the presence of  $H_2S^*$ , no sulfur\* is present in the residual cystine (or cysteine).

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# THE INHIBITION OF CHOLINESTERASE BY PHYSOSTIGMINE AND PROSTIGMINE

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It has been generally assumed that the inhibition of cholinesterase by physostigmine and prostigmine is non-competitive. This is implicit in the equation given by Matthes (1) relating the degree of inhibition to the concentration of physostigmine, for it contains no term for substrate concentration, an omission justified only if the inhibition is non-competitive. However, it should be noted, as Matthes himself points out, that his equation fits only a certain range of the data, and that the discrepancies with low physostigmine concentration are probably not to be accounted for by experimental error. In any case, as Lineweaver and Burk (2) have made clear, the type of inhibition cannot be determined from this relationship.

Some years later Easson and Stedman (3) reached the tentative conclusion that inhibition by prostigmine is non-competitive on the indirect evidence that, if enzyme and inhibitor are allowed to reach equilibrium before the substrate is added, hydrolysis of the latter, provided a sufficient excess is present, proceeds at a constant rate. Their theoretical relationship between prostigmine concentration and the degree of inhibition is essentially the same as that of Matthes: there is assumed to be a reversible but non-competitive combination between 1 molecule of inhibitor and one active center of enzyme to form an inactive compound. They state that it is necessary to leave prostigmine in contact with the enzyme for at least 9 hours before measuring the inhibition in order to insure equilibrium, and in their experiments they apparently left the mixture for about 24 hours. Similarly Roepke (4) states that approximately 8 hours are required for physostigmine and prostigmine to reach an equilibrium with the enzyme. Using an indicator method, he measured the rate of hydrolysis of varying concentrations of acetylcholine by a cholinesterase preparation which had been mixed with the drug and left overnight in the ice box. When the reciprocals of the velocities thus obtained were plotted against the reciprocals of the substrate concentrations (*cf.* Lineweaver and Burk (2)), it was found that the points lay upon two straight lines which did not cut the  $1/v$  axis at the same point, typical of non-competitive inhibition. On the other hand he failed to confirm Easson and Stedman, finding that the rate



of hydrolysis very slowly decreased on addition of substrate, and concluded that these drugs are probably also competitive inhibitors.

An equilibrium reached only after 8 or 9 hours would seem to be, in the case of these drugs, of only slight physiological importance, for the action in the body begins within a few minutes and is usually over within a couple of hours. Effects obtained immediately after the drug was mixed with the enzyme were accordingly investigated with an electrometric titration method slightly modified from that of Alles and Hawes (5) and described elsewhere (Eadie (6)). Serum was kept in a stoppered glass tube through which bubbled nitrogen. This precaution was taken because otherwise blanks with serum alone were found to become alkaline at a measurable rate, presumably from loss of carbon dioxide. Dog serum was used as the source of enzyme, 1 cc. being added to the titration mixture, the total volume of which was 25 cc. The alkali used was 0.01 *N* NaOH, and the temperature was 36.5°.

*Existence of Early Equilibria*—The usual criterion for the existence of an equilibrium is taken to be the constancy of the rate of hydrolysis of acetylcholine. Three sets of conditions, however, must be distinguished. (1) When the initial concentration of acetylcholine is sufficiently high so that it remains practically constant during the course of the experiment (20 minutes), the amount hydrolyzed per minute will remain constant. (2) With lower acetylcholine and moderately low inhibitor concentrations, the rate will follow the usual logarithmic law for monomolecular reactions. (3) It will be shown later that the inhibition is competitive; because of this the degree of inhibition will be greater, the lower the substrate concentration. Hence with low acetylcholine and relatively high inhibitor concentrations, the rate will fall off more quickly than predicted by the monomolecular equation. This departure, however, will not occur until an appreciable amount of substrate has been hydrolyzed.

When the experiments were analyzed, it was found that with high acetylcholine concentrations plotting the NaOH used against time yielded a straight line; plotting  $\log a/(a - x)$  against time also yielded a straight line not only here but with lower acetylcholine concentrations, the only exceptions being when the inhibitor concentrations were very high, and even in these cases the divergence did not occur before 15 minutes or so. Fig. 1 illustrates the first of these cases, and also shows that the new equilibrium became established within 5 minutes. In most cases examined it took about half this time.

Velocities, accordingly, were calculated by fitting the data to the logarithmic equation by the method of least squares. The velocity constant thus obtained was multiplied by the initial acetylcholine concentration to get the actual initial velocity, a correction being made for hydrolysis due to OH ion.

All experiments were rejected in which the variance of estimate (*i.e.* the sum of the squares of deviation from a straight line divided by the degrees of freedom) exceeded an arbitrary value which was fixed at  $1.0 \times 10^{-6}$ . In the case of the experiments in which a departure from linearity occurred towards the end, only the earlier points were used, provided, of course, that they met the preceding criterion.

*Effect of Varying Substrate Concentration*—The difference between competitive and non-competitive inhibition can be determined only by experiments with varying substrate concentration (2, 7). The relationship

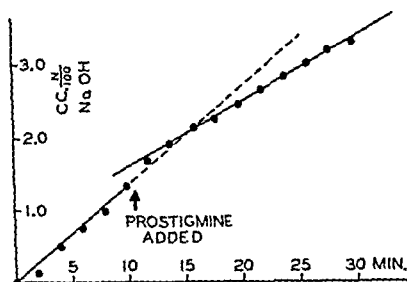


FIG. 1. The effect of the addition of prostigmine on the hydrolysis of acetylcholine by dog serum. Experimental points are marked. The dotted line shows how hydrolysis would have proceeded if prostigmine had not been added. It will be seen that a new rate becomes established about 5 minutes after the addition of prostigmine.

between velocity of hydrolysis and substrate concentration is given by the equation, modified from Michaelis and Menten (8)

$$v = \frac{Vx}{x + K_p}$$

where  $v$  is the initial velocity,  $x$  the molar substrate concentration, and  $V$  the value of  $v$  when  $1/x$  is 0.  $K_p$  stands for  $K_M (1 + f^n/K_I)$  where  $K_M$  and  $K_I$  are the enzyme-substrate and enzyme-inhibitor dissociation constants respectively,  $f$  the molar concentration of inhibitor, and  $n$  the number of molecules of inhibitor combining with 1 molecule (or active center) of enzyme. In the absence of inhibitor  $K_p$  reduces to  $K_M$ . In non-competitive inhibition the value of  $V$  is decreased, and in competitive inhibition that of  $K_p$  is increased.

The equation in the form given is not very easy to deal with, and Lineweaver and Burk (2) suggested using  $1/x$  and  $1/v$  as the variables; so that it becomes

$$\frac{1}{v} = \frac{1}{V} + \frac{K_p}{V} \cdot \frac{1}{x}$$

When plotted, this gives a straight line of slope  $K_p/V$  cutting the  $1/v$  axis at  $1/V$ . Competitive inhibition is now characterized by a change in slope due to a change in  $K_p$  (but not in  $V$ ), and no change in the point of interception on the  $1/v$  axis. Fig. 2 shows the data from a typical experiment with physostigmine plotted in this way, and Fig. 3 one with prostigmine. It is clear that in each case the data indicate competitive inhibition.

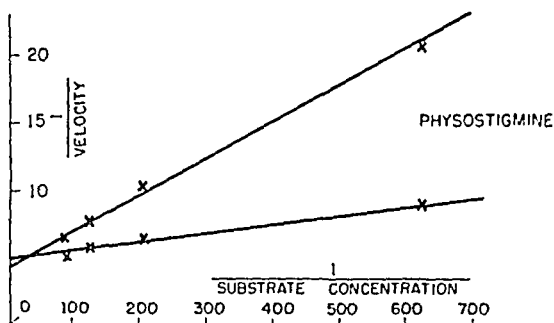


FIG. 2. The effect of substrate concentration on the velocity of hydrolysis of acetylcholine by dog serum in the presence (upper curve) and absence (lower curve) of physostigmine,  $1 \times 10^{-6}$  M.

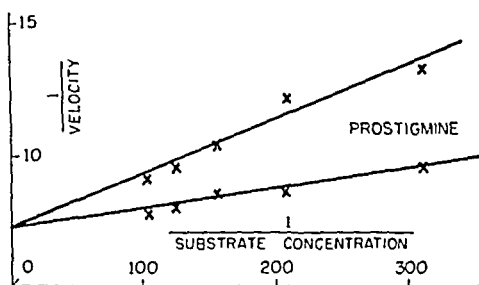


FIG. 3. The effect of substrate concentration on the velocity of hydrolysis of acetylcholine by dog serum in the presence (upper curve) and absence (lower curve) of prostigmine,  $1.5 \times 10^{-7}$  M.

The data show enough variation to make a statistical analysis advisable. For this purpose the equation as just given is not very satisfactory, particularly if we are interested in the value of  $K_p$ . We may, however, rewrite it in the form

$$v = V - K_p c$$

where  $c$  is the velocity constant; *i.e.*,  $v/x$ . This form has the advantage that the slope,  $K_p$ , is now the regression coefficient, and the two constants

have been placed in separate terms.  $V$  can be shown to be equal to  $\bar{v} - K_p \bar{c}$ , where  $\bar{v}$  and  $\bar{c}$  are the mean values of these variables.

The question whether the variation in the values obtained for  $K_p$  in different experiments may be attributed to the experimental error of the individual determinations can be answered by calculating  $F$ , the ratio of the two variances involved. In each of the three cases (acetylcholine alone,

TABLE I  
*Constants Obtained by Successive Poolings of Data on Dog Serum*

$K_M$ ; acetylcholine	$K_p$	
	Physostigmine, $2.4 \times 10^{-7} M$	Prostigmine, $1.5 \times 10^{-7} M$
0.00209	0.00527	0.00416
0.00126	0.00474	0.00304
0.00130	0.00347	0.00282
0.00120	0.00367	0.00281
0.00120	0.00443	0.00289
0.00136	0.00459	0.00268
0.00167	0.00457	0.00272
0.00168		0.00263
0.00166		0.00264
0.00165		0.00263
0.00160		0.00265
0.00156		0.00260
0.00154		0.00260
0.00154		0.00267
0.00171		0.00269
0.00170		
0.00170		
0.00170		
0.00168		
0.00169		
0.00168		
0.00167		
0.00166		
Standard error of mean..... 0.00014	0.00049	0.00014

and with physostigmine and prostigmine)  $F$  was found to be below the level of significance; experimental error is therefore sufficient to account for the differences. The test also indicates that pooling of data is permissible. Table I shows the values of the constants obtained by successive poolings, and illustrates the more or less rapid approach to a fairly stable value. The most probable value is the final one in each series. The

standard error of the mean is also given. The difference in  $K_p$  produced by the addition of physostigmine and prostigmine was found to be highly significant by the  $t$  test. This is characteristic of competitive inhibition.

$V$  is a value obtained by extrapolation, and therefore subject to greater errors than  $K_p$ . Calculation of  $F$ , however, gave values well beyond the 1 per cent level of significance, indicating that the differences between individual estimations are greater than might be expected from the errors involved. This is not surprising, since  $V$  depends not only on the characteristics of the enzyme but also on the amount of it per cc. of serum. Significant alterations in  $V$  were also found to occur on storage. We are therefore not justified in pooling the data to arrive at the most probable value of  $V$ , but must compare averages. For each batch of serum there was usually one determination of  $V$  without inhibitor, and several with. Each determination was made from several titrations, usually three to five. For the sera used for the physostigmine experiments the values of  $V$  without and with inhibitor were averaged, each being weighted according to the number of titrations involved in its determination; the standard deviations were also calculated. In the absence of physostigmine the average  $V$  was  $0.207 \pm 0.47$  (six experiments), and in the presence of  $2.4 \times 10^{-7}$  M inhibitor  $0.233 \pm 0.032$  (seven experiments). There is no significant difference between these values, and in any case the second is higher than the first rather than lower. For the sera used in the prostigmine experiments the values were without prostigmine  $0.178 \pm 0.028$  (six experiments) and with prostigmine ( $1.5 \times 10^{-7}$  M)  $0.176 \pm 0.013$  (fifteen experiments). Here again there is no significant difference. There is thus no evidence of any change in  $V$  as would have occurred with non-competitive inhibition. Statistical analysis thus confirms the conclusion that the inhibition is competitive.

The best value for the enzyme-substrate dissociation constant is  $0.0017 \pm 0.0001$ . The calculation of the inhibitor-enzyme dissociation constants must be postponed until we know the value of  $n$ , the number of molecules of inhibitor combining with 1 of enzyme.

*Effect of Inhibitor Concentration*—If our interest is in the relationship of the velocity to the inhibitor concentration with constant substrate concentration, it is convenient to rearrange the terms of the Michaelis equation and simplify by substituting new constants to get

$$1/v = a + bf^n$$

If  $n = 1$ , which is usual, then the reciprocals of the velocities plotted against the inhibitor concentrations should form a straight line. Data for physostigmine are plotted in Fig. 4 and for prostigmine in Fig. 5. Only with lower concentrations of the inhibitor do the points approach a straight line.

If, however,  $n = 2$ , the points should fall on the curves shown in Figs. 4 and 5, and it is obvious that they fit them fairly closely.

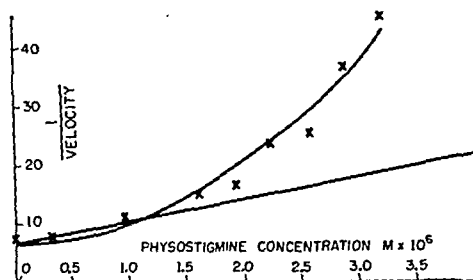


FIG. 4. The effect of physostigmine concentration on the hydrolysis of acetylcholine (0.008 M) by dog serum. If 1 molecule of inhibitor combines with 1 of enzyme, the points should fall on the straight line; if 2 molecules of inhibitor, on the curved line.

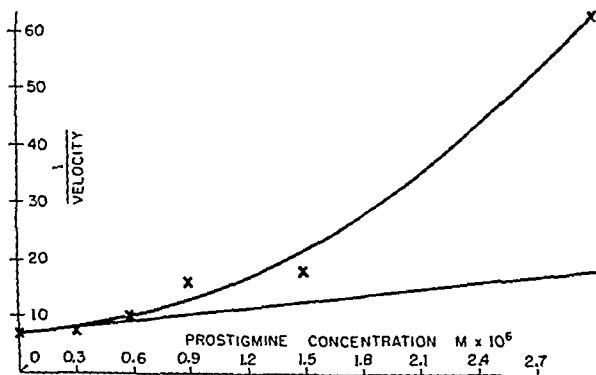


FIG. 5. The effect of prostigmine concentration on the hydrolysis of acetylcholine (0.008 M) by dog serum. If 1 molecule of inhibitor combines with 1 of enzyme, the points should fall on the straight line; if 2 molecules of inhibitor, on the curved line.

The data have been analyzed statistically by fitting them, by the methods of multiple regression, to the equation

$$1/v = A + Bf + Cf^2$$

The standard partial regression coefficient for the first power of  $f$  was found to be negative and not significant; that for the second power, positive and significant. Deviations from linear regression were also found to be signif-

icantly greater than from curved regression. From this it may be concluded that  $1/v$  varies as the second power of  $f$ , or that 2 molecules of these inhibitors combine with 1 of enzyme.

Since Easson and Stedman believe that their experiments indicate a combination of 1 molecule of prostigmine with 1 of enzyme, it is of interest to examine their data from this point of view. The results given in their Table VII have accordingly been plotted in Fig. 6, the (approximately) 24 hour figures in the column headed "Titration" being used as a measure of the velocity (two figures missing from this table can be supplied from the data in Table VIII). No correction was made for the difference in "time after mixing," since, as they point out, its magnitude is exceedingly small. The straight line and curve have the same significance as in Figs. 4 and 5. Here also the experimental points fit the curve very closely throughout the

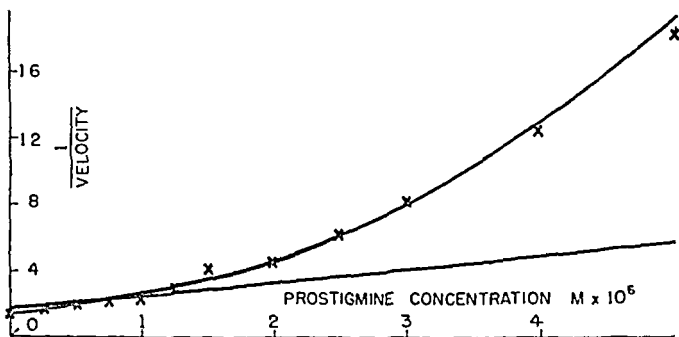


FIG. 6. The effect of prostigmine concentration on the hydrolysis of acetylcholine by dog serum (data of Easson and Stedman). If 1 molecule of inhibitor combines with 1 of enzyme, the points should fall on the straight line; if 2 molecules of inhibitor, on the curved line.

whole range, and do not fit the straight line except at lower concentrations, again indicating that the inhibiting combination is between 2 molecules of drug and 1 of enzyme.

*Inhibitor-Enzyme Dissociation Constants*—The enzyme-inhibitor dissociation constants may now be calculated from the data in Table I. They are  $3.3 \times 10^{-14}$  for physostigmine and  $3.6 \times 10^{-14}$  for prostigmine.

The standard deviation for these values cannot be calculated exactly, because  $\rho$ , the correlation coefficient between  $K_M$  and  $(K_p - K_M)$ , is unknown. Maximum possible values for the standard deviations will be obtained if  $\rho = -1$ ; these are  $3.14 \times 10^{-14}$  and  $4.33 \times 10^{-14}$  respectively; the true values are unquestionably less than this. Another estimate of the range may be obtained by using the ranges of  $K_M$  and  $K_p$  given by their standard deviations. This leads to a range of  $K_i$  for physostigmine of  $4.54 \times 10^{-14}$  to  $2.47 \times 10^{-14}$ , and for prostigmine of  $5.40 \times 10^{-14}$  to  $2.61 \times 10^{-14}$ .

*Comment*

The type of inhibition has a bearing on the question whether the physiological effects are due to enzyme inhibition. In general, concentrations used in the test-tubes are likely to be greater than those reached at the point of action in the body, and, if inhibition is non-competitive, to produce the same degree of it would require the same concentration. On the other hand, if it is competitive, the extent of inhibition will depend also on the substrate concentration. Putting it in another way, we may say that the affinity of the enzyme for physostigmine or prostigmine is  $10^{11}$  times its affinity for its substrate. These experiments therefore make it possible to explain the *in vivo* action on the basis of that *in vitro*.

The combination of 2 molecules of inhibitor with 1 of enzyme means that the number of enzyme molecules blocked increases with the square of the inhibitor concentration, rather than with the first power. It is thus possible to attain practically complete inhibition much more readily.

I am deeply indebted to the Laboratory of Experimental Statistics, State College, Raleigh, North Carolina, for advice on statistical analysis, to Hoffmann-La Roche, Inc., for the gift of 1 gm. of prostigmine salts, and to Miss J. L. Smith and to Miss M. J. Stalleup for assistance in carrying out the experiments.

## SUMMARY

1. Mixtures of cholinesterase with physostigmine or prostigmine reach an equilibrium within a few minutes after being mixed.
2. The inhibition of the enzyme by these drugs is competitive.
3. 2 molecules of inhibitor combine with 1 molecule (or active center) of enzyme.
4. The inhibitor-enzyme dissociation constants are approximately  $3 \times 10^{-14}$  for physostigmine and  $2 \times 10^{-14}$  for prostigmine, the cholinesterase being that of dog serum at  $36.5^\circ$ . Under the same conditions the enzyme-substrate dissociation constant was found to be  $1.7 \times 10^{-3} \pm 0.1$ .

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# THE EFFECT OF HEXOSES AND PENTOSES ON THE FORMATION IN VITRO OF PHOSPHOLIPID BY BRAIN TISSUE AS MEASURED WITH RADIOACTIVE PHOSPHORUS\*

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With the aid of radioactive phosphorus it has been demonstrated that surviving slices of liver, kidney, and brain and brain homogenates are capable of synthesizing phospholipids from inorganic phosphate (1, 2). The term "synthesis" as applied here refers to the incorporation of inorganic phosphate into the phospholipid molecule, which means, rigidly defined, the formation of two ester bonds of phosphate.

It is well known that the oxygen consumption of brain preparations remains nearly constant for long periods of time when placed in a Ringer's medium containing glucose, whereas in a glucose-free Ringer's medium the oxygen uptake decreases rapidly (3). These observations suggested the present investigation of the effects of glucose and other carbohydrates on phospholipid formation by surviving brain preparations.

## EXPERIMENTAL

Rats of both sexes weighing 14 to 18 gm. (6 to 8 days old) were used in the following experiments. The preparation of brain slices, brain homogenates, and the bicarbonate-Ringer's solution containing  $P^{32}$  in the form of inorganic phosphate has been described elsewhere (2). The amount of radio-phosphorus present in each bath gave from  $5 \times 10^5$  to  $1 \times 10^6$  counts per minute on the Geiger-Müller counter employed in this laboratory. The methods used for the extraction of phospholipid and for the determination of its radioactivity have also been recorded (2).

0.2 cc. of sugar solution was added to the 5 cc. of Ringer-bicarbonate medium contained in each reaction flask. The sugar solutions were prepared so that the added 0.2 cc. yielded the recorded concentrations of sugar in the reaction medium.

## Results

*Effect of Hexoses and Pentoses*—The effect of various hexoses and pentoses on the recovery of radiophospholipid in surviving brain slices and homogenates is shown in Table I. The results are expressed as percentages of the

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inorganic radiophosphorus initially present in the bath recovered as radiophospholipid per gm. of wet tissue.

A striking increase in the recovery of radiophospholipid was observed when glucose, fructose, mannose, and galactose were added to the Ringer's medium containing brain slices. When pentoses were added, no such effect was found. The amounts of radiophospholipid recovered in the presence of glucose, mannose, and galactose were approximately equal. In the presence of these hexoses, the recovery was about 5 times as great as that in the

TABLE I

*Effect of Hexoses and Pentoses on Recovery of Radiophospholipid in Brain Slices and Brain Homogenates*

All values are expressed as per cent of the added  $P^{32}$  recovered as radiophospholipid per gm. of wet tissue.

Experiment No.	Tissue preparation	Period of incubation <i>hrs.</i>	Sugar*				
			No sugar	Glucose	Fructose	Mannose	Galactose
1	Slices	2	0.54	2.6	1.8	2.4	
		2	0.68	2.7	2.1	2.3	
		2	0.48	2.7			
2	Slices	2	0.57	2.5	1.5	2.4	2.3
		2	0.66	2.5	1.4		2.2
		2	0.55	2.3	2.0		
3	Slices			Rhamnose	Arabinose		
		2	0.52	0.24	0.56		
		2	0.30	0.38	0.42		
		2	0.41	0.35	0.53		
				Glucose	Fructose		
4	Homogenate	4	0.18	0.19	0.30	0.35	0.27
		4	0.23	0.25	0.25	0.31	0.26
		4	0.20	0.29	0.25	0.29	0.25

\* In each case an amount of sugar was added to make its concentration in the bath 0.02 M.

control samples which contained no *added* sugar. When fructose was added, the recovery of radiophospholipid was 3 times that of the control. No appreciable change in the pH of the bicarbonate-Ringer's medium was observed during the course of the experiment whether or not it contained added glucose.

Although it has been demonstrated previously that brain homogenates form appreciable amounts of phospholipid from inorganic phosphate (2), this type of preparation failed to show an increased recovery of radiophospholipid when hexoses were added.

*Effect of Variation of Glucose Concentrations*—Table II shows that in 2 hour experiments glucose concentrations differing by a factor of 100, *i.e.* from a concentration of 0.001 to 0.1 M, caused the same 4- to 5-fold increase above the control in the recovery of radiophospholipid. In experiments prolonged to 4 hours (Fig. 1) the lowest glucose concentration, namely 0.001 M,<sup>1</sup> did not have so great an effect as the higher concentrations. It would

TABLE II

*Effect of Glucose Concentration on Recovery of Radiophospholipid in Brain Slices*

All values are expressed as per cent of the added P<sup>32</sup> recovered as radiophospholipid per gm. of wet tissue. Period of incubation, 2 hours.

Control	Glucose concentration			
	0.001 M	0.005 M	0.02 M	0.10 M
0.63	2.5	2.5	3.0	2.7
0.73	2.5	2.5	3.0	2.7
0.48	2.1	2.9	2.6	2.5

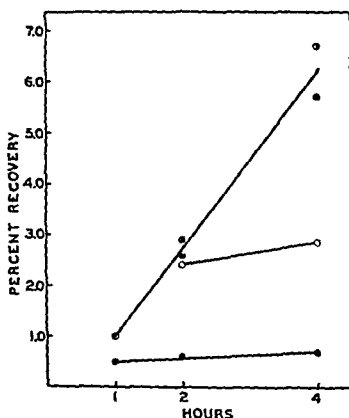


FIG. 1. The effect of various concentrations of glucose on the recovery of radiophospholipid at various time intervals. ● control (no glucose added); ○ 0.001 M glucose; ◐ 0.005 M glucose; ◑ 0.02 M glucose.

appear in this case that the glucose present had been completely utilized by the end of 2 hours and therefore failed to show stimulating effects on radiophospholipid recovery at the 4 hour interval.

<sup>1</sup> It can be calculated from Kerr and Ghantus' (4) figures for dog and rabbit cerebrum that the amount of glucose plus glycogen present in 300 mg. of brain slices is equivalent to about one-half the amount of glucose which has to be added to the 5 cc. samples of Ringer's medium to yield a concentration of 0.001 M.

As regards the higher glucose concentrations (0.005 and 0.02 M), it was found that the recovery of radiophospholipid after 4 hours was about twice the 2 hour value. It can be seen from Fig. 1 that a nearly straight line is obtained when radiophospholipid recovered in the presence of 0.005 and 0.02 M glucose is plotted against time. This suggests that the rate of incorporation of inorganic radiophosphorus into phospholipid remains constant under the influence of glucose for at least 4 hours. In the absence of glucose, however, the rate of radiophospholipid formation decreases considerably after the 1st hour. It is interesting to note that a similar situation has been observed for the  $O_2$  uptake by brain slices in the presence and absence of glucose. When glucose is present, the rate of  $O_2$  consumption is nearly constant for at least 3 hours, whereas in the absence of glucose the rate of  $O_2$  uptake decreases after the 1st hour (3).

*Effect of Glucose and Fructose in  $N_2$  Anaerobiosis*—In the experiments recorded in Table III, slices were incubated in a bicarbonate-Ringer's medium

TABLE III

*Effect of  $N_2$  Anaerobiosis on Recovery of Radiophospholipid in Brain Slices*

All values are expressed as per cent of the added  $P^{32}$  recovered as radiophospholipid per gm. of wet tissue. Period of incubation, 2 hours.

Control in $O_2$	Control in $N_2$	Glucose (0.02 M) in $N_2$	Fructose (0.02 M) in $N_2$
0.45	0.075	0.24	0.14
0.50	0.19	0.25	0.13
0.54	0.056	0.15	0.078

that had been saturated with a gas mixture consisting of 95 per cent  $N_2$  and 5 per cent  $CO_2$ . After the addition of the slices, the flask was thoroughly flushed with the same  $N_2$ - $CO_2$  gas mixture, tightly stoppered, and placed in the thermostat.

The exclusion of oxygen resulted in a decreased recovery of radiophospholipid in brain slices. This is in agreement with the results obtained for liver and kidney under similar conditions (5). When glucose and fructose were added to brain slices incubated under anaerobic conditions, no significant increase in the recovery of radiophospholipid was observed. It appears that the stimulating effect of these sugars occurs only in the presence of oxygen. The variability of the results obtained in the  $N_2$  experiments suggests that the small amounts of radiophospholipid recovered under these conditions were due to traces of oxygen still present after the flasks were flushed with nitrogen, rather than to anaerobic phospholipid synthesis.

*Penetration of Phosphate into Slice*—The slow penetration of phosphate observed in the brain *in vivo* does not apply to thin brain slices suspended in

the bicarbonate-Ringer's solution. In the latter case it is reasonable to assume that the inorganic phosphate of the medium enters into rapid equilibrium with the inorganic phosphate of the extracellular spaces.

The amount of phospholipid  $P^{32}$  recovered is a function of (a) the rate of phospholipid formation and (b) the specific activity of the precursors at the site of phospholipid formation. If it be assumed that phospholipid formation takes place in the extracellular spaces or on the outer surface of the cell, phospholipid is formed from an ultimate precursor, namely inorganic phosphate of the extracellular spaces, whose specific activity reaches a maximum value very rapidly and remains at this maximum, or close to it, throughout the course of the experiment. So long as the penetration of inorganic phosphate into the extracellular spaces is a rapid process, the specific activity of the inorganic phosphate in the extracellular spaces cannot be influenced by hexoses. Radiophospholipid, therefore, is formed at the expense of an ultimate precursor whose specific activity is the same in the presence and in the absence of hexoses. Increased recovery under these conditions can mean only an increased rate of formation of phospholipid or of a phosphorus-containing phospholipid precursor.<sup>2</sup> Therefore, if the incorporation of inorganic phosphate into the phospholipid molecule or into a phospholipid precursor takes place in the extracellular spaces, the effect of hexoses observed in the present investigation represents a true stimulation in the rate of formation of phospholipid or of a phosphorus-containing phospholipid precursor.

If, however, the formation of phospholipid occurs within the cell, then the rapidity with which the labeled inorganic phosphorus (the ultimate precursor of the radiophospholipid measured in these experiments) penetrates into the cell, or, more specifically, to the exact site of phospholipid formation, will be a decisive factor in determining the recovery of radiophospholipid. It is possible that under the influence of an added substance the rate of penetration of inorganic phosphorus is either increased or decreased, and the specific activity of the inorganic phosphorus at the exact site of phospholipid formation thereby altered. Under these conditions the recovery of radiophospholipid would be increased or decreased even though the rate of phospholipid formation remained constant.

The above considerations apply to all quantitative studies with tissues, *in vitro* as well as *in vivo*, in which the effect of various substances upon the

<sup>2</sup> The experiments as carried out here do not distinguish between an increased rate of formation of phospholipid and an increased rate of formation of a phosphorus-containing phospholipid precursor. An increased rate of formation of such a precursor would increase its specific activity; in this case an increased recovery of radiophospholipid would be obtained even if the rate of phospholipid formation were not increased.

rate of formation of a compound is measured by means of isotopes as tracers. This is particularly true in experiments of short duration (6). Changes in the rate of penetration of the tracer element under the influence of an added substance may cause an increased recovery of the tracer element in the compound studied. Hence the mere increase or decrease in the recovery of the tracer element in the compound studied, by itself, cannot be interpreted to mean an increase or decrease in the rate of formation of the compound under investigation.

These considerations led to the following experiments: 300 mg. of brain slices were placed in flasks containing 5 cc. of either a glucose-free radioactive Ringer's solution or a glucose-containing radioactive Ringer's solution. The amount of radiophosphorus present in each bath gave from  $1 \times 10^4$  to  $5 \times 10^4$  counts per minute. At intervals of 1, 2, and 3 hours a few slices were removed from each bath, washed twice with non-radioactive

TABLE IV

*Effect of Glucose on Penetration of  $P^{32}$  into Brain Slices*

All values are expressed as per cent of the added  $P^{32}$  recovered in the brain slices per gm. of wet tissue.

Period of incubation	Control	Glucose
hrs.		
0	Negligible	Negligible
1	26, 22, 28, 24 (25)	37, 42, 52, 47 (45)
2	24, 27, 24, 30 (26)	55, 54, 66, 66 (60)
3	26, 27, 29, 26 (26)	58, 64, 68 (63)

The figures in parentheses are averages.

Ringer's solution, weighed, and their  $P^{32}$  content determined.<sup>3</sup> The results are expressed as the percentages of the added  $P^{32}$  recovered per gm. of tissue (Table IV). In the experiments recorded in Table V, which were carried out in a manner similar to those above, the total and inorganic phosphorus contents of brain slices were measured after a 2 hour incubation in glucose-free and glucose-containing media.

No significant difference was observed in the total phosphorus content of slices that had been incubated in (a) a glucose-free bicarbonate-Ringer's solution and (b) a glucose-containing bicarbonate-Ringer's solution (Table V). The values found at the 2 hour interval agreed closely with the 0 time values. The amount of inorganic phosphorus found in brain slices

<sup>3</sup> Negligible amounts of radioactivity were found in brain slices that were placed in the radioactive Ringer's solution and immediately thereafter removed (0 time interval).

incubated for 2 hours in a glucose-containing Ringer's solution was only slightly less than that found in slices kept in glucose-free Ringer's solution. The values for inorganic phosphorus found in the 0 time experiments agreed closely with those obtained in brain slices incubated for 2 hours in glucose-free Ringer's solution.

The recovery of  $P^{32}$  in the brain slices kept in a glucose medium is greater than in those suspended in a glucose-free medium (Table IV). This was the case at all three time intervals examined. Maximum values for  $P^{32}$  recovery were observed at 1 hour in the control experiments, whereas in the presence of glucose an increase in the recovery of  $P^{32}$  was obtained between 1 and 2 hours.

Under the influence of glucose the recovery of phospholipid  $P^{32}$  in brain slices was increased about 3-fold after 1 hour, about 5-fold after 2 hours, and about 10-fold after 4 hours of incubation (Fig. 1). If these increased recoveries are to be accounted for solely by increased penetration

TABLE V

*Inorganic and Total Phosphorus Content of Brain Slices*

All values are expressed as per cent of the wet weight of the tissue.

	Period of incubation	Control	Glucose
	<i>hrs.</i>		
Inorganic P	0	0.029, 0.032	
	2	0.031, 0.032	0.025, 0.025
Total P	0	0.21, 0.22	
	2	0.18, 0.19	0.22, 0.21

of  $P^{32}$  and the resulting increase in specific activity of the phospholipid precursor at the site of phospholipid formation, then it would be necessary for the specific activity of this precursor to be increased by about the same factors, namely 3-, 5-, and 10-fold, at the respective time intervals. The results on the penetration of  $P^{32}$  (Table IV) suggest that the increased recovery of radiophospholipid under the influence of glucose is not due solely to increased penetration of inorganic phosphate. Further evidence for this view is provided in the following experiments.

Brain slices were incubated for 45 minutes in a bicarbonate-Ringer's solution containing  $P^{32}$  in the form of inorganic phosphate. The slices were then carefully removed from the radioactive bath, and in order to free the slices of adhering  $P^{32}$  they were dipped twice (for 1 minute each time) into separate non-radioactive bicarbonate-Ringer's solutions. These slices were then divided into three portions and treated as follows: The radiophospholipid of one portion (Sample A) was determined immediately. The radiophospholipid of a second portion of slices (Sample B) was deter-



mined after the slices were incubated for 2 hours in a non-radioactive bicarbonate-Ringer's solution. The third portion (Sample C) was incubated for 2 hours in a non-radioactive bicarbonate-Ringer's solution that contained glucose in 0.02 M concentration, and its radiophospholipid was then determined. The average recoveries of radiophospholipid per gm. of wet tissue, expressed as the percentage of the  $P^{32}$  added to the bath, were 0.17 per cent for Sample A, 0.19 per cent for Sample B, and 0.52 per cent for Sample C.

These results show that the phospholipid  $P^{32}$  content of the slices is greatly increased by incubation in the non-radioactive medium containing glucose (see Samples A and C). But incubation in the non-radioactive bicarbonate-Ringer's solution containing no glucose failed to increase the radiophospholipid recovery (see Samples A and B).

The increased penetration of inorganic phosphate (non-radioactive) into the slice under the influence of glucose (Sample C) should decrease the specific activity of the inorganic phosphate inside the slice and presumably at the site of phospholipid formation. Hence, if the effect of glucose on the recovery of radiophospholipid (Tables I and II) were due solely to an increased penetration of inorganic phosphate, the recovery of radiophospholipid should be less in the case of Sample C (glucose in a non-radioactive medium) than in the case of Sample B (non-radioactive medium with no glucose). The fact that the recovery of radiophospholipid was increased by glucose (Sample C), despite the dilution of the  $P^{32}$  within the slice by increased penetration of non-radioactive inorganic phosphate, leads to the conclusion that an actual increase in the rate of phospholipid or phospholipid precursor formation had occurred.

#### DISCUSSION

The stimulating effects of hexoses upon the rate of formation of phospholipid or of a phosphorus-containing phospholipid precursor may be explained by (a) the provision by the hexoses of increased oxidative energy for the formation of phospholipid or of a phosphorus-containing phospholipid precursor or (b) the direct formation of a phospholipid precursor from the added hexoses. At present no evidence exists for the latter case.

Taurog *et al.* (5) have concluded from a study of the effects of various respiratory poisons that a close relation exists between phospholipid formation and respiration. The well known effects of glucose in stimulating oxygen consumption of brain slices, together with the observations recorded here, seem to be in line with such a view.

The samples of radiophosphorus used in this investigation were prepared in the Berkeley cyclotron by members of the Radiation Laboratory under the direction of Professor E. O. Lawrence, to whom our thanks are due.

## SUMMARY

1. The conversion *in vitro* of inorganic  $P^{32}$  to phospholipid  $P^{32}$  by surviving brain slices is greatly increased by the addition of the hexoses glucose, galactose, mannose, and fructose. This increase in recovery of radiophospholipid is interpreted as being due to an increased rate of formation of phospholipid or of a phosphorus-containing phospholipid precursor.

2. The addition of pentoses failed to increase the recovery of radiophospholipid.

3. The stimulating effect of the hexoses upon the recovery of radiophospholipid is abolished when tissue organization is disrupted by homogenization.

4. The stimulating effect of the hexoses upon the recovery of radiophospholipid does not occur under anaerobic conditions.

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# REACTION OF NINHYDRIN WITH ASCORBIC ACID AND OTHER ENDIOL COMPOUNDS

## DECARBOXYLATION OF DEHYDROASCORBIC ACID

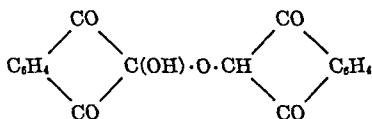
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When solutions of ninhydrin and ascorbic acid are mixed at room temperature, a very insoluble crystalline precipitate forms. The reaction occurs very quickly when the mixture is heated. 0.5 mg. of ascorbic acid mixed with 5 mg. of ninhydrin per ml. of solution and heated over a flame for 2 to 3 minutes and quickly cooled gives a definite precipitate which has the same properties as that formed at room temperature. Reductone and dihydroxymaleic acid react with ninhydrin to give similar precipitates.

Ruheman<sup>1</sup> has shown that when ninhydrin is reduced with hydrogen sulfide a crystalline precipitate of hydrindantin,  $C_{18}H_{10}O_6$ , melting at  $236^\circ$ , is formed, to which he assigns the structure,



Its formation depends upon the reduction of ninhydrin to the corresponding alcohol and the addition of this alcohol to ninhydrin. Ruheman demonstrated that hydrindantin gives a blue color with amino acids, as does ninhydrin. It also dissolves in strong bases to give a deep blue color which disappears upon shaking with air or oxygen. Hydrindantin can be recovered from the strongly alkaline solutions by immediate acidification, but not after destruction of the blue color by oxidation. Hydrindantin also dissolves in alkali-carbonate solutions to give a deep red color which is not discharged by aeration. Hydrindantin is recovered by acidification of the carbonate solution.

The precipitates formed as a result of the reaction of ninhydrin with ascorbic acid and other endiol compounds possessed all of the properties of Ruheman's hydrindantin. Freshly prepared samples turned red at about  $200^\circ$  as described by Ruheman, but both our preparations and that

<sup>1</sup> Ruheman, S. J., *J. Chem. Soc.*, 99, 792, 1306 (1911).

made according to Ruheman's directions did not show this color change after standing for a year in a desiccator. When our preparations were mixed with hydrindantin prepared according to Ruheman, the mixtures melted at 235–237°.

Equimolecular amounts of ascorbic acid and ninhydrin were mixed in approximately 10 per cent solution in water and allowed to stand overnight at room temperature. The precipitate formed was removed by filtration, washed several times on the filter with cold water, and dried in an oven at 100°. Carbon and hydrogen determinations on this preparation showed C 66.58, H 3.23; theory for hydrindantin,  $C_{18}H_{10}O_6$ , C 67.08, H 3.10. The molecular weight of this preparation by the ebullioscopic method with dioxane as solvent was 331 (theory for hydrindantin, 322).

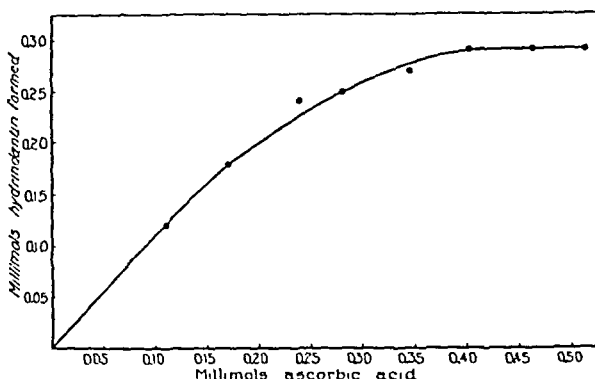


FIG. 1. Formation of hydrindantin in the reaction of 0.58 mm of ninhydrin with ascorbic acid at room temperature for 48 hours.

As shown in Fig. 1 approximately 2 moles of ninhydrin per mole of ascorbic acid yielded a maximum amount of precipitate, and in the presence of excess ascorbic acid the moles of hydrindantin formed were equivalent to one-half the moles of ninhydrin present. This is in accord with a reaction involving the oxidation of 1 mole of ascorbic acid by 1 mole of ninhydrin, and the subsequent reaction of 1 mole of reduced ninhydrin with 1 mole of ninhydrin to form 1 mole of hydrindantin, as described by Ruheman<sup>1</sup> in the reaction of ninhydrin with hydrogen sulfide.

The slow evolution of gas was observed from mixtures of ninhydrin and ascorbic acid upon standing at room temperature. This gas was identified as carbon dioxide. Since ascorbic acid is oxidized quickly by ninhydrin and the evolution of carbon dioxide slowly follows this oxidation, it seemed probable that the carbon dioxide arose from the oxidation product of ascorbic acid. The oxidation of ascorbic acid by ninhydrin apparently stops at the stage of dehydroascorbic acid, since mixtures of dehydroascor-

bic acid and ninhydrin give no precipitate of hydrindantin. The experiments outlined below seem to indicate that the carbon dioxide arises from the decomposition of dehydroascorbic acid, presumably by decarboxylation after hydrolysis of the lactone bridge.

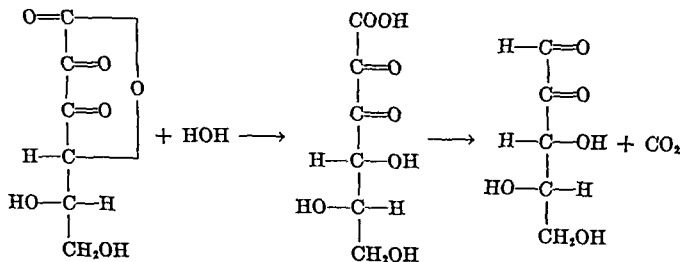
The amount of carbon dioxide evolved after oxidation of ascorbic acid by ninhydrin was determined as follows: The ascorbic acid and ninhydrin were dissolved in 25 ml. of water and placed in a vessel arranged for the passage of gas through the solution. The vessel containing the mixture and a similar one containing water were connected and placed in a water bath at constant temperature. A stream of purified hydrogen was bubbled first through the vessel containing only water, next through the ninhydrin-ascorbic acid mixture, and then through concentrated sulfuric acid, drierite, and a weighed tube of ascarite in succession. The ascarite tube contained a layer of drierite after the layer of ascarite. Evolved  $\text{CO}_2$  was swept into the ascarite tube and weighed. Known solutions of carbonate were acidified in the reacting vessel and theoretical recovery of  $\text{CO}_2$  was obtained. Care was exercised in all experiments to continue the passage of gas (with interruptions for weighing) until the ascarite tube showed constant weight.

From 0.60 mm of ascorbic acid treated with 2.30 mm of ninhydrin (excess) at  $60^\circ$  0.65 mm of  $\text{CO}_2$  was evolved in 26 hours.

From 1.18 mm of ascorbic acid (excess) and 1.13 mm of ninhydrin 0.54 mm of  $\text{CO}_2$  was given off in 26 hours. When mixtures were held at  $30^\circ$ , evolution of  $\text{CO}_2$  was only one-third complete in 24 hours.

Solutions of dehydroascorbic acid were prepared by exact titration of ascorbic acid with iodine, and carbon dioxide evolution was determined at  $60^\circ$  as above. From 0.58 mm of dehydroascorbic acid 0.50 mm of  $\text{CO}_2$  was obtained in 24 hours and 0.59 mm in 72 hours, at which time evolution of the gas had ceased. In another experiment 1.52 mm of dehydroascorbic acid gave 1.53 mm of  $\text{CO}_2$  in 58 hours.

The decarboxylation of dehydroascorbic acid presumably involves hydrolysis of the lactone bridge followed by loss of carbon dioxide and production of *l*-xylosone, as shown in the accompanying formulas.



Preliminary observations showed the presence of a substance which reduces cold Benedict's solution in solutions of dehydroascorbic acid that had evolved carbon dioxide and from which hydrogen iodide had been removed with silver carbonate. These solutions did not reduce indophenol after treatment with hydrogen sulfide, indicating the absence of dehydroascorbic acid. An attempt is being made to characterize the substance.

The decarboxylation of dehydroascorbic acid undoubtedly plays a part in the destruction of the compound in solutions at ordinary temperature. It appears reasonable to suppose that at least a part of the metabolism of ascorbic acid in the body involves oxidation to dehydroascorbic acid followed by decarboxylation.

#### SUMMARY

1. Ninhydrin readily oxidizes compounds containing the endiol group.
2. Ninhydrin oxidizes ascorbic acid to dehydroascorbic acid, which then loses a molecule of carbon dioxide, presumably through decarboxylation.
3. The decarboxylation of dehydroascorbic acid is undoubtedly partly responsible for the instability of solutions of the compound. The metabolism of ascorbic acid in the body may, in part, involve oxidation to dehydroascorbic acid followed by decarboxylation.

# A MICROBIOLOGICAL METHOD FOR THE DETERMINATION OF *p*-AMINOBENZOIC ACID

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The discovery by Woods (1) of the antisulfonamide activity of *p*-aminobenzoic acid, the finding by Rubbo and Gillespie (2, 3) of its essential nature for the growth of *Clostridium acetobutylicum*, and the reports of Ansbacher (4) and Martin and Ansbacher (5) of its function as an animal vitamin have combined to make *p*-aminobenzoic acid the subject of extensive investigation (6-13).

Because of the growing biological importance of *p*-aminobenzoic acid, a method for its quantitative determination is desirable. In spite of much experimental work there is no immediate prospect that *p*-aminobenzoic acid can be measured by either chemical or animal assay. It therefore appears probable that microbiological methods will have to be relied upon.

The development of the microbiological assay method reported here is based on the studies of Underkofler, Bantz, and Peterson (14) on the nutrition of *Acetobacter suboxydans*. Their findings were extended in this laboratory (15) and additional information obtained on the nutrition of this organism. From this was developed a simple, accurate microbiological assay for the determination of *p*-aminobenzoic acid.

## EXPERIMENTAL

*Organism*—The test organism employed is *Acetobacter suboxydans*,<sup>1</sup> which is carried on yeast extract-glycerol-agar (16) (0.5 per cent Bacto-yeast extract, 5 per cent glycerol, pH 6). Stock cultures are transferred at monthly intervals and are refrigerated in the interim. Inoculum for assays is prepared by transfer from the stock culture to a flask of the basal medium (Table I) to which has been added 0.05  $\gamma$  of *p*-aminobenzoic acid. The inoculum culture is incubated at 30° for 24 hours prior to use and is transferred daily from the preceding liquid cultures, being returned to a stock culture at weekly intervals.

*Basal Medium*—The composition of the basal medium is given in Table I. This formula is twice the desired final concentration. All chemicals em-

<sup>1</sup> Cultures of this organism may be obtained from the American Type Culture Collection, Georgetown University Medical School, Washington, where it is listed as No. 621.



ployed are of the highest purity. Smaco "vitamin-free" casein hydrolysate<sup>1</sup> is sufficiently free of *p*-aminobenzoic acid to serve as the source of nitrogen. The reaction of the medium is adjusted to pH 6.0  $\pm$  0.1. The resulting basal medium may appear opalescent or cloudy. It is desirable to filter the medium through hardened filter paper, Filter Cel, or Seitz E. K. pad to avoid formation of a precipitate during autoclaving.

*Assay Procedure*—5 cc. of basal medium and dilutions of material under test made up to 5 cc. with distilled water are placed in 50 cc. Erlenmeyer flasks.<sup>3</sup> Similarly, a set of reference flasks is prepared containing from 0.01 to 0.1  $\gamma$  of *p*-aminobenzoic acid. Blanks containing no *p*-aminobenzoic acid are included. The flasks are plugged with cotton and autoclaved at 15 pounds pressure for 15 minutes. After cooling they are inoculated with a suspension of *Acetobacter suboxydans*. A 24 hour culture

TABLE I  
*Basal Medium for p-Aminobenzoic Acid Assay (Undiluted)*

Casein hydrolysate.....	0.6 gm.
Glycerol.....	10.0 "
Tryptophane.....	20 mg.
Cystine.....	15 "
K <sub>2</sub> HPO <sub>4</sub> .....	100 "
KH <sub>2</sub> PO <sub>4</sub> .....	100 "
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	40 "
NaCl.....	2 "
FeSO <sub>4</sub> ·7H <sub>2</sub> O.....	2 "
MnSO <sub>4</sub> ·2H <sub>2</sub> O.....	2 "
Calcium pantothenate.....	200 $\gamma$
Nicotinic acid.....	200 "
Distilled water to.....	100 cc.
pH adjusted to 6.0	

grown as described above is centrifuged and washed twice in 10 cc. of sterile saline solution. The washed cells are resuspended in 15 cc. of saline solution. 1 drop (approximately 0.05 cc.) of the resulting suspension per flask serves as the inoculum. The flasks are incubated at 30° for 48 hours. Following incubation, 10 cc. of water are added to each flask to dilute the culture suitably for measurement of turbidity and the contents are thoroughly mixed by shaking. Growth response to incre-

<sup>2</sup> This purified casein hydrolysate may be obtained from the Research Laboratories, S. M. A. Corporation, Chagrin Falls, Ohio. Several other purified casein hydrolysates have also been found satisfactory.

<sup>3</sup> *Acetobacter suboxydans* is an obligate aerobe. Small Erlenmeyer flasks (50 cc.) permit heavier growth of 10 cc. cultures than do the usual bacteriological culture tubes.

ments of *p*-aminobenzoic acid is shown in Fig. 1 and is determined by measurement of turbidity with a photoelectric colorimeter. Such a standard curve is constructed by plotting the colorimeter readings against the concentration of *p*-aminobenzoic acid. The *p*-aminobenzoic acid content of material under test is then read from the standard curve. Samples are tested at dilutions which permit calculation of results at several levels

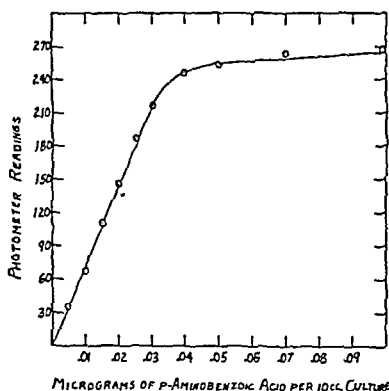


Fig. 1. Growth response of *Acetobacter suboxydans* No. 621 to *p*-aminobenzoic acid

TABLE II  
*p*-Aminobenzoic Acid Content of Materials at Varying Assay Levels

Yeast extract			Urine			Peptone		
Amount per assay flask	<i>p</i> -Aminobenzoic acid		Amount per assay flask	<i>p</i> -Aminobenzoic acid		Amount per assay flask	<i>p</i> -Aminobenzoic acid	
	Found	Content		Found	Content		Found	Content
mg.	$\gamma$	$\gamma$ per gm.	cc.	$\gamma$	$\gamma$ per cc.	mg.	$\gamma$	$\gamma$ per gm.
0.75	0.025	33	2.0	0.025	0.012	40	0.015	0.375
0.50	0.018	37	1.5	0.019	0.012	30	0.012	0.399
0.25	0.009	36	1.0	0.013	0.013	20	0.008	0.400
Average .....		35.3			0.012			0.391

of the curve within accurate assay limits. The final value is an average obtained from the figures yielded at various levels (Table II). The extreme upper and lower portions of the curve are not reliable for assay work, and only that portion of the curve which is linear is used for the calculation of assay values.

*Preparation of Sample for Assay*—Materials which are soluble in water are assayed without further treatment. Insoluble materials such as food-

stuffs, grains, and animal tissues are finely divided, extracted with 10 to 20 volumes of water at 15 pounds pressure for 30 minutes, and centrifuged or filtered. Such treatment has yielded maximum assay values. Body fluids require special handling, since many were found to inhibit growth of the test organism. The inhibitory action of blood, spinal fluid, ascitic fluid, and urine is readily overcome by autoclaving. To illustrate, blood is prepared for assay as follows: The sample is laked with an equal volume of distilled water and autoclaved. 3 volumes of water are added to the autoclaved blood while hot, and the sample shaken for several minutes to disperse and extract the protein precipitate. The suspension is centrifuged

TABLE III  
*p*-Aminobenzoic Acid Content of Various Materials

	$\gamma$
Brewers' yeast, per gm. . . . .	102
Yeast extract (Bacto), per gm. . . . .	40
Rice bran concentrate, " cc. . . . .	1.1
Liver extract (Lilly, No. 343), per gm. . . . .	5.0
Fresh calf liver, per gm. . . . .	0.2
Meat extract, per gm. . . . .	1.3
Peptone, per gm. . . . .	0.4
Blood (human), per cc. . . . .	0.035
Spinal fluid (human, 37 pooled), per cc. . . . .	0.25
Urine, per cc. . . . .	0.015
Cow's milk, per cc. . . . .	0.15
Wheat germ, " gm. . . . .	1.0
Alfalfa meal, " " . . . . .	2.0
Corn-meal, per gm. . . . .	0.3
Molasses, " cc. . . . .	0.01
Rolled oats, per gm. . . . .	0.33
Wheat middlings, per gm. . . . .	0.52

and the supernatant filtered to give a clear extract suitable for assay. Other body fluids may be prepared for assay by the above procedure. They do not show inhibition and give assay values in good agreement at various levels.

*Occurrence of p-Aminobenzoic Acid*—The distribution of *p*-aminobenzoic acid in various foodstuffs is indicated in Table III. While only a limited number of materials have been assayed, it is apparent that *p*-aminobenzoic acid is widely distributed. This distribution is, in general, similar to that exhibited by the various B vitamins. Compared with other materials assayed, brewers' yeast is by far the richest source of *p*-aminobenzoic acid. All body fluids examined contained appreciable amounts of *p*-aminobenzoic acid. The presence of *p*-aminobenzoic acid in liver extract, fresh liver, and meat extract indicates its probable presence in most body tissues.

*Specificity of p-Aminobenzoic Acid*—In order to test the specificity of the growth response of *Acetobacter suboxydans* to *p*-aminobenzoic acid, a group of compounds either derived from or related to *p*-aminobenzoic acid was quantitatively assayed for growth support of *Acetobacter suboxydans*. The activity of these *p*-aminobenzoic acid derivatives and related compounds is given in Table IV. In no instance was the activity found comparable to that of *p*-aminobenzoic acid. Substitution, whether in the amino or carboxyl group, sharply reduces or completely removes biological activity. Compounds referred to in Table IV as inactive either support

TABLE IV  
Activity of *p*-Aminobenzoic Acid Derivatives and Related Compounds\*

Compound	<i>p</i> -Aminobenzoic acid activity
	per cent
<i>p</i> -Aminophenylacetic acid.....	1.6
<i>p</i> -Aminoethyl benzoate.....	0.06
<i>p</i> -Aminophenylglycine.....	Inactive
<i>p</i> -Acetaminobenzoic acid.....	"
<i>p</i> -Aminobenzoyldiethylaminoethanol.....	2.0
<i>p</i> -Nitrobenzoylglycine†.....	0.35
<i>p</i> -Chloroacetylbenzoylglycine†.....	0.20
<i>p</i> -Glycylbenzoylglycine†.....	0.04
<i>p</i> -Glycylaminobenzoic acid†.....	9.0
<i>o</i> -Aminobenzoic acid.....	Inactive
<i>m</i> -Aminobenzoic ".....	"
2-Aminonicotinic acid.....	"
<i>o</i> -Aminobenzoylformic anhydride.....	"
<i>o</i> -Hydroxybenzoic acid.....	"

\* *p*-Aminobenzoic acid is considered to have 100 per cent activity. Solutions of the various compounds were sterilized by filtration through Seitz filters.

† These compounds were kindly supplied by Dr. D. W. Woolley, the Rockefeller Institute, New York.

growth of the test organism only in large concentrations (1000 or more times that of *p*-aminobenzoic acid) or have no demonstrable activity. The majority of the derivatives has activity of such slight degree as to be of no practical importance in microbiological assay of *p*-aminobenzoic acid.

*Accuracy of Assay*—*p*-Aminobenzoic acid added to various test materials is recovered in a range of 90 to 105 per cent. This together with the agreement of assay values calculated from different dosage levels of test samples (Table II) is indicative of the accuracy of the method. As computed from recovery tests and values obtained from different quantities of test samples the accuracy of the method seems comparable to that exhibited by most microbiological methods; viz.,  $\pm 15$  per cent.

*Remarks*

The proposed microbiological assay for *p*-aminobenzoic acid meets the requirements of a satisfactory assay method since (a) no growth occurs in the basal medium in the absence of *p*-aminobenzoic acid. The basal medium does, however, support good growth when *p*-aminobenzoic acid is added, the quantity of growth so obtained being equivalent to that obtainable with natural products. (b) Assay values obtained with small amounts of test material are in good agreement with those obtained by the use of larger supplements. (c) It is possible to recover accurately any known amount of *p*-aminobenzoic acid which has been added to the product under examination. (d) *p*-Aminobenzoic acid supplied in large excess is not inhibitory. (e) Compounds related to or derived from *p*-aminobenzoic acid have little or no biological activity, indicating that the method possesses a high degree of specificity.

## SUMMARY

A rapid, accurate, and specific microbiological assay method for the determination of *p*-aminobenzoic acid has been developed based upon the growth response of *Acetobacter suboxydans* to *p*-aminobenzoic acid.

The authors are greatly indebted to Professor L. A. Underkofler for his kindness in supplying information on the nutrition of *Acetobacter suboxydans*.

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# THE QUANTITATIVE DETERMINATION OF FACTOR V BY MEASUREMENT OF NITRITE PRODUCED BY *HEMOPHILUS INFLUENZAE*

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The V factor content of animal tissues became of interest to workers in the field of nicotinic acid metabolism following the discovery by Lwoff and Lwoff that either coenzyme I or coenzyme II could replace this factor in the metabolism of *Hemophilus influenzae* and *Hemophilus parainfluenzae* (1). Recently a report that nicotinamide nucleoside, a component of coenzyme, can also function as the V factor has appeared (2).

Some success has attended the use of influenza bacilli in the determination of the V factor by measurement of the growth stimulus which is afforded these organisms, in appropriate culture media, by the introduction of material containing the factor (1, 3). The technique suffers marked limitation, however, in that it depends on turbidity as an indication of the extent of growth of the test organism, an index which may be completely unreliable in those instances in which insoluble or deeply pigmented materials containing the V factor have been added to the culture media. On the other hand, if non-bacterial turbidity and interfering pigments are avoided by preliminary extraction of the V factor, such extraction may be incomplete and may result in partial destruction of the factor, which in its reduced state is extremely labile and is affected by the concentration of dilute acid employed in the extraction process (4, 5). Measurement of the end-point of growth of *Hemophilus influenzae* with increasing dilution of the test material, according to the method of Vilter, Vilter, and Spies (6), is not suited for studies when determination of the V factor in absolute units is desired (7).

In certain microbiological methods of assay, considerable success has followed the use of a stable metabolite, such as lactic acid, in lieu of turbidity, as a measure of the growth of the test organism (8). In a study of the metabolic products of the influenza bacillus we have found that the property of nitrate reduction exhibited by this organism (7) can be employed readily and successfully as a quantitative expression of its metabolic activity, and that the nitrite produced in a standard medium can be used reliably as a measure of the V factor content of added test materials. Moreover, the sensitivity of the method used for the quantitative determination of nitrite permits the performance of the test on such small aliquots of

culture material that turbidity or color resulting from added test substances does not appreciably interfere. In this technique preliminary extraction of the V factor is unnecessary, and simplification of the procedure is achieved by adding test material, obtained with sterile precautions, directly to the standard medium.

### *Materials and Methods*

*Preparation of Media*—Media proposed by various workers for the growth of *Hemophilus influenzae* were tested. The medium found best for the quantitative determination of the V factor was composed of 2.0 per cent Difco proteose-peptone, 0.6 per cent sodium chloride, and 0.2 per cent sodium or potassium nitrate as a source of nitrite. The media were autoclaved for 20 minutes at 116° and autoclaved rabbit, sheep, or human blood was added after sterilization in a concentration of 1 per cent.

*Preparation of Inoculum*—Standard inocula were prepared in 5 cc. of a stock broth composed of 2.0 per cent proteose-peptone, 0.6 per cent sodium chloride, 0.1 mg. of autoclaved hemin, and 0.06  $\gamma$  of purified coenzyme as the V factor. Nitrate was omitted in order to prevent transfer of nitrite with the inoculum. Organisms carried on heated blood agar by weekly transfers were inoculated into a tube of stock broth and incubated overnight at 37°. After three transfers in stock broth the organisms were ready for use. When 0.1 cc. of such a culture was inoculated into 10 cc. of medium, no multiplication or reduction of nitrate occurred unless the medium contained V factor.

A number of strains of *Hemophilus influenzae* have been used in the development of this technique. Although strains were found to differ markedly in the rate and total amount of nitrite produced in a given medium, when the same strain was used for the production of a standard curve and the determination of the V factor content of a given test material, results were obtained which checked closely with determinations of the V factor made with other strains.

*Preparation of Coenzyme Standard*—Coenzyme I, suitable for establishing V factor curves, was prepared from yeast by the method of Williamson and Green (9). A solution containing 1.5 mg. per cc. of a preparation of coenzyme previously dried to constant weight was passed through a Berkefeld filter. An aliquot of the sterile filtrate was set up in various dilutions, and, by means of the technique described, compared with a standard sample of coenzyme kindly supplied by Dr. B. J. Jandorf of the Department of Biological Chemistry, Harvard Medical School. The activity of this preparation had been previously determined enzymatically by comparison with a sample of coenzyme of which the purity had been established by analysis for phosphorus and by measurement of the extinc-

tion coefficient at  $3400 \text{ \AA}$ , following reduction with dithionine. The filtrate, standardized by the nitrate reduction technique, was diluted to contain  $15 \gamma$  of coenzyme per cc., delivered aseptically in 0.5 cc. quantities into sterile tubes, frozen and dried *in vacuo*, and stored in a desiccator over calcium chloride at  $0^\circ$ . Frequent assays have demonstrated that coenzyme preparations treated and stored in this manner are stable for many months.

#### EXPERIMENTAL

*Determination of Nitrite Produced by Hemophilus influenzae*—For the determination of nitrite the technique devised by Shinn (10) has been found very satisfactory. In this method the color produced by the diazotization of sulfanilamide and subsequent coupling of the diazotized compound to N(1-naphthyl)ethylenediamine is a measure of the nitrite in the reaction mixture. From a 1.0 cc. pipette 0.1 cc. of the culture was delivered into a colorimeter tube containing 8.5 cc. of water. The interior of the pipette was then washed two to three times with small quantities of the material in the tube to insure complete delivery. This was followed successively by 1.0 cc. of 0.2 per cent sulfanilamide, 0.2 cc. of 0.1 per cent N(1-naphthyl)ethylenediamine dihydrochloride, and 0.2 cc. of 6 N hydrochloric acid. The deep red color which developed upon coupling of diazosulfanilamide with N(1-naphthyl)ethylenediamine was maximum within 15 to 20 minutes and was stable for at least 24 hours. The color intensity was read in a photoelectric colorimeter with Filter 520 (maximum transmission).

*Coenzyme Standard Curve*—Six concentrations of sterile coenzyme, 0.0, 0.0075, 0.01, 0.025, 0.03, and 0.0375  $\gamma$ , respectively, were added in triplicate to 9 cc. of media described above in 50 cc. Erlenmeyer flasks, and sufficient sterile water added to bring the volume to 10 cc. The flasks were next inoculated with 0.1 cc. of the standard culture of *Hemophilus influenzae* previously described and incubated for 48 hours at  $37^\circ$ . 0.1 cc. of the 48 hour culture was then added to 8.5 cc. of water and the nitrite determined as described above.

The range of nitrite concentration which can be determined by diazotization and production of color by coupling is a narrow one. This technical limitation is further increased by the fact that the amount of nitrite produced by *Hemophilus influenzae* is relatively great for small increments of coenzyme. For these reasons it has been found best, in most instances, to limit the amount of culture taken for nitrite analysis to 0.1 cc. However, with certain strains of *Hemophilus influenzae* which reduce nitrates less effectively, 0.2 to 0.5 cc. of the culture media may be required for an accurate nitrite determination. When 1 cc. or less of the medium is taken for analysis, even great turbidity of the culture medium due to



added test material does not interfere significantly with the photoelectric determination of color intensity.

Although standard curves of the nitrite produced with a given strain of *Hemophilus influenzae* are fairly constant from day to day, enough variation occurs to make it necessary to set up a new series of standards for each set of coenzyme test materials. Since *Hemophilus influenzae* responds indifferently to coenzyme I and coenzyme II (11), determinations of the total V factor have been expressed in terms of coenzyme I. In Fig. 1 are recorded the results of a typical standard curve obtained by plotting photometric density of color obtained from the nitrite produced by *Hemophilus influenzae* in a standard medium against increments of added coenzyme I.

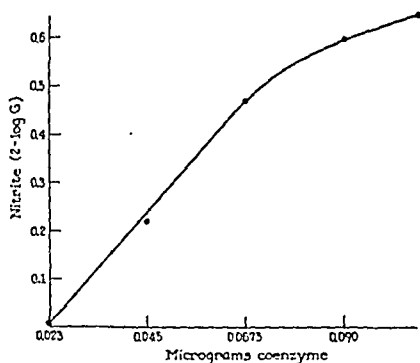


FIG. 1. Production of nitrite by *Hemophilus influenzae* in response to increments of coenzyme in a medium free of V factor.

*Preparation of Blood for V Factor Determination*—Blood was drawn under sterile precautions into sterile test-tubes containing ammonium and potassium oxalates in the proportions suggested by Wintrobe (12). The solution of oxalates was filtered to insure sterility and dried in the tubes at 65° before use. Preliminary dilution and laking of freshly drawn blood were performed with a volumetric 0.5 cc. pipette carefully wiped free of blood on the outer surface with sterile gauze and rinsed into 4.5 cc. of sterile water. From this 1:10 dilution further dilutions were made. Amounts of diluted blood representing usually 0.005 to 0.001 cc. of whole blood were added to 9 cc. of media, in duplicate flasks, and as a rule a second series of similar dilutions was prepared and set up in the same manner. The volume of media and test material was brought to 10 cc. with sterile water and the flasks were inoculated with 0.1 cc. of the standard inoculum and incubated for 48 hours. At the end of the incubation period, 0.1 cc. of each culture was mixed with 8.5 cc. of distilled water

according to the manner described for the preparation of the standard coenzyme curve and the nitrite determined colorimetrically. The V factor content of the blood dilution was read directly from the standard curve and expressed finally as micrograms of coenzyme per cc. of erythrocytes. The erythrocyte volume was determined on an aliquot of 1 cc. of blood in a Wintrobe hematocrit tube (12). Previous methods for the microbiological determination of coenzyme have called for the heating of laked blood before it is added to the media. In this study the results of a number of experiments have shown that heating the blood to 85° caused no change in the growth response of *Hemophilus influenzae*; hence was considered unnecessary.

*Recovery Experiments with Known Concentrations of Coenzyme*—In Table I are given a number of values obtained when known amounts of coenzyme I were added to media containing blood with previously determined V factor content. The close agreement between the expected and

TABLE I  
*Quantitative Recovery of V Factor*

The results are measured in micrograms.

Total V factor in blood sample	Coenzyme I added	Total V factor expected	Total V factor observed
0.037	0.023	0.060	0.060
0.028	0.023	0.051	0.050
0.019	0.023	0.042	0.039
0.012	0.015	0.027	0.030

observed values for the V factor concentration under these conditions shows that the method may be applied not only to the V factor concentration in blood, but to the standardization of coenzyme preparations as well, when a coenzyme standard of known purity is available for comparison.

*V Factor Concentration in Normal Blood*—Values for the V factor content of normal erythrocytes are given in Fig. 2. Most values have fallen between 50 and 70  $\gamma$  of V factor, expressed as coenzyme I, per cc. of red cells, although a few values as low as 40  $\gamma$  have been obtained in individuals, who, from lack of history and clinical signs to the contrary, were considered normal. A survey of a fairly wide range of diseases, including amyotrophic lateral sclerosis, cardiac decompensation, cirrhosis of the liver, hypothyroidism, hypertension, lupus erythematosus, nephritis, pneumonia, rheumatic fever, scarlatina, and thyrotoxicosis, has revealed no consistent deviation from this range. Vilter, Vilter, and Spies, by another technique (6), have reported low V factor values in pellagra, diabetic

acidosis, and leucemia. Studies of the V factor concentration in the blood and tissues of pellagrins will appear in a later communication.

*V Factor Concentration in Blood Following Ingestion of Nicotinic Acid and Nicotinamide*—Increased erythrocyte concentration of the V factor following the ingestion of nicotinic acid has been reported by Kohn (3) and by Axelrod and associates (13). With the nitrate reduction technique a marked increase in the concentration of V factor in blood, following the ingestion of nicotinic acid, has been consistently observed. Three persons, whose diets for several months had been adequate in nicotinic acid, were

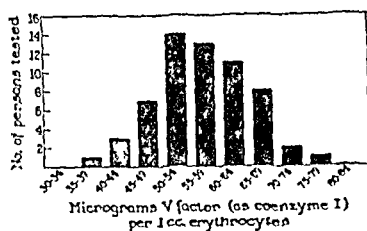


FIG. 2. The range of concentration of V factor in normal blood

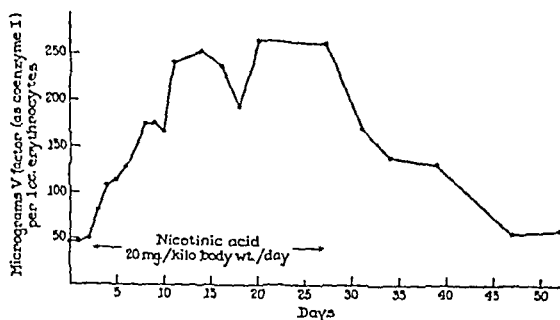


FIG. 3. Increased concentration of V factor in blood following the daily ingestion of 20 mg. of nicotinic acid per kilo of body weight.

placed on large daily doses of nicotinic acid, amounting to approximately 20 mg. per kilo of body weight per day. Within 48 to 72 hours a marked rise in blood V factor was observed in each case. A curve for one of these individuals, covering a period of nicotinic acid ingestion of 25 days, is reproduced in Fig. 3.

For purposes of comparing the response of blood V factor to nicotinamide with that observed following nicotinic acid ingestion, three other normal individuals were given 20 mg. of nicotinamide per kilo of body weight per day and the concentration of blood V factor followed for 15 days. No

appreciable rise in blood V factor was observed. At the end of this period nicotinamide was stopped and 20 mg. of nicotinic acid per kilo of body weight per day begun. A prompt rise in blood V factor was observed. The results of this experiment are recorded in Fig. 4. The lack of V factor increase in the blood following the ingestion of nicotinamide is quite surprising in view of the fact that nicotinic acid and nicotinamide are regarded as equivalent in the prevention of nicotinic acid deficiency states and in the treatment of the deficiency when once induced (14). Further work is required before the significance of this observation can be properly assessed and related to the total pattern of nicotinic acid metabolism. The possi-

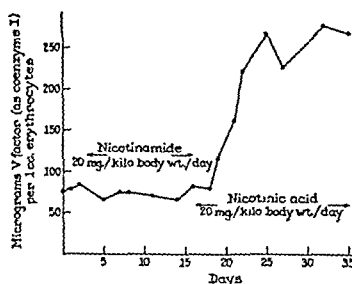


FIG. 4. Increased concentration of V factor in blood following the ingestion of nicotinic acid after a period without change during the ingestion of nicotinamide.

bility that separate metabolic pathways for the two compounds exist must be entertained.

#### SUMMARY

The nitrite produced by *Hemophilus influenzae* in a basal medium containing sodium or potassium nitrate can be used as a quantitative measure of the V factor content of added blood, provided all other essentials for the growth of this organism are present in optimal concentration.

With this technique, the V factor content of normal human erythrocytes has been shown to fall between 40 and 70  $\gamma$  per cc. expressed as coenzyme I.

The ingestion of nicotinic acid produced an immediate rise in V factor concentration in the blood of normal individuals. Nicotinamide, on the other hand, ingested in equivalent quantities, produced no appreciable change in the concentration of V factor in the blood.

The possibility that separate metabolic pathways for nicotinic acid and nicotinamide exist is being further investigated.

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# THE CHEMICAL DETERMINATION OF TOCOPHEROLS IN MUSCLE TISSUE

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Aside from its importance in reproduction tocopherol (vitamin E) is essential in maintaining the structural and functional integrity of skeletal muscle in certain species of animals. The method of biological assay with female rats is in itself evidence that the amounts of tocopherol required for the latter purpose are smaller than those needed to prevent reproductive failure; this was also indicated by the early observations of Ringsted (1). Inasmuch as the time-consuming and expensive biological assay has a wide range of variability (2), its successful application to the determination of the still smaller amounts of tocopherol associated with muscle function assumes statistical proportions. Without quantitative information on the tocopherol content of muscle under various conditions a proper appreciation of its rôle cannot be achieved.

Several chemical methods (3-6) and two physical methods (7, 8) have been proposed for the quantitative determination of tocopherol. Although used chiefly to measure the content of oils and oil concentrates (9, 10), some of these chemical methods have, on occasion, been employed, either in their original or in modified form, to estimate the amount of tocopherol in tissues, blood serum, urine, and feces.

A review of these methods indicated that the colorimetric procedure of Emmerie and Engel (3) which has recently been applied to human serum (11) is the most sensitive and that perhaps it could be applied to muscle tissue. The destructive effect of even mild saponification (12) was confirmed; saponification should therefore be avoided if possible, since it does not accomplish the removal of the principal interfering substances, vitamin A and carotene. Correction is made for them by indirect methods which are long and not entirely satisfactory. They have been removed by adsorption on floridin XS but in our hands<sup>1</sup> this adsorbent proved to be less specific than its proponents recommended it to be (13), even with pure

\* The experimental data are taken from a dissertation submitted by Henry B. Devlin to the Faculty of the Graduate College of the State University of Iowa in partial fulfilment of the requirements for the degree of Doctor of Philosophy, July, 1942.

<sup>1</sup> Various adsorbents were kindly supplied by the Floridin Company, Warren, Pennsylvania.

solutions; the requirements of an adsorbent might reasonably be more demanding with a muscle extract than with an extract of serum.

At this stage of the study several aids became known. One of these was the Parker and McFarlane procedure (14) in which the double bonds which reduce ferric ion are destroyed by 85 per cent sulfuric acid. When this was applied to pure solutions of tocopherol, there was no loss.

Another valuable aid was the Waring blender for shredding and extracting tissue (15). The use of this with an alcohol-benzene solvent and the Parker-McFarlane technique gave reasonably repeatable results but the large amounts of fat were troublesome especially in vacuum distillation; turbidity also interfered occasionally.

The next advance was the use of the foaming solvent described by Moore and Ely (16). Since this solvent gave the highest yield of carotenoids from plant material, it was reasonable to expect that it might also accomplish complete extraction of the unsaponifiable material from animal tissue, along with a minimum of fat. This expectation was justified by the recovery experiments mentioned later.

When these several modifications were included, two further difficulties arose. One was the inconstancy of the colorimeter readings with time and the other, more puzzling, was the lack of difference between the tocopherol content of muscle from animals on a vitamin E-deficient diet and that of muscle from normal animals on stock diet.

All colorimeter readings showed an increase with time, but with tissue extracts of deficient animals the readings increased so rapidly as to be meaningless. This could be caused by the presence of additional chromogenic substances in the muscle extracts from deficient animals.

Morgulis and coworkers (17) had found that dystrophic rabbit muscle contained abnormally large amounts of cholesterol; this is also true for muscle and brain tissue from vitamin E-deficient rats.<sup>2</sup> The question arose as to what influence, if any, cholesterol might have on the determination of vitamin E. The introduction of a new solvent, glacial acetic acid,<sup>3</sup> for the Emmerie and Engel reagent, facilitated the study of this problem. With this solvent also the reagent<sup>4</sup> becomes insensitive to light and remains stable for a long time. Results of experiments on solutions of pure cholesterol can be summarized briefly. Pure cholesterol in Skellysolve E, 117–127° (SSE), gave increased readings with time after treatment by the Parker-McFarlane procedure, whether glacial acetic acid or alcohol was the solvent for the reagent, but cholesterol in SSE, without this treatment,

<sup>2</sup> Heinrich, M. R., unpublished observations from this laboratory.

<sup>3</sup> Personal communication from Merck and Company, Inc.

<sup>4</sup> 250 mg. of ferric chloride and 500 mg. of  $\alpha, \alpha'$ -bipyridine in a liter of glacial acetic acid.

did not respond to the reagent in either solvent. Mixtures of cholesterol and tocopherol in SSE, on addition of the glacial acetic acid reagent, showed a time change in color development; when treated by the Parker-McFarlane procedure, the color increased rapidly; when not so treated, the color rapidly faded to zero.

The Parker-McFarlane treatment does not remove cholesterol or destroy its reactivity with the reagent, but it does bring about a chemical alteration in cholesterol as indicated by a pink color produced by the sulfuric acid and dispelled by the potassium hydroxide, and by a precipitate appearing in treated solutions of cholesterol on addition of glacial acetic acid. Tissue extracts, wheat germ oil, and cholesterol solutions after this treatment still gave a positive Liebermann-Burchard test for sterols. According to Parker and McFarlane (14) the treatment of vegetable oils by sulfuric acid destroys the sterols. Apparently the possible complicity of cholesterol in this colorimetric determination of vitamin E has not been suspected.

It was obvious that vitamin E and cholesterol or the related interfering substance must be separated before this method could be applied to animal tissues. Precipitation of the latter by digitonin was not successful; in pure solution  $\alpha$ -tocopherol appeared to be destroyed by the procedure.

Selective adsorption of cholesterol on purified<sup>5</sup> super-sorb (florisil) accomplished the separation. From pure solutions of tocopherol only, recoveries were 94, 98, 102, and 103 per cent, in the presence of cholesterol 102, 99, 89, and 96 per cent.

Finally it was discovered that reactive material entered the extracts from pure gum rubber tubing and stoppers; when an all-glass system was used, repeatable results were obtained.

The complete procedure as finally used was as follows: From 10 to 25 gm. of muscle from the hind legs of rats were placed in the blender with approximately 200 cc. of the foaming solvent.<sup>6</sup> After 5 minutes agitation the liquid was centrifuged, and the residue subjected to a second, third, and fourth treatment; further extraction proved to be unnecessary. Water was added to the combined supernatant liquids to about 40 per cent alcohol concentration and the mixture was shaken gently. Alcohol was subsequently added to break the emulsion. The alcohol-water phase was a fine emulsion and contained no chromogenic substances; the petroleum ether phase plus the four washings, containing the unsaponifiable matter, was evaporated to dryness under diminished pressure in the presence of nitrogen. The residue was taken up in 15 cc. of benzene<sup>7</sup> and two 5

<sup>5</sup> As directed by Emmerie and Engel for floridin XS (13).

<sup>6</sup> Purified (14) Skellysolve B (SSB) and absolute alcohol in a ratio of 8.5:10; the latter was purified by distillation and gave no test with the reagent.

<sup>7</sup> Reagent benzene is satisfactory; it gave no test with the reagent.



cc. aliquots were individually passed through  $30 \times 12$  mm. adsorption columns of purified super-sorb. Each column was washed with four 5 cc. portions of benzene, after which the benzene was removed from the filtrates by evaporation on a boiling water bath in a stream of nitrogen.

One of the aliquots was then treated by the Parker-McFarlane technique as follows: 10 cc. of SSE were added, the solution was transferred to a glass-stoppered centrifuge tube, 2 cc. of 85 per cent sulfuric acid were added, and the tube was shaken and then centrifuged for 2 minutes. As much as possible of the supernatant SSE solution was decanted into a similar tube, 5 cc. of 2 per cent potassium hydroxide were added, and the mixture was shaken and then centrifuged for 10 minutes. To 5 cc. of the supernatant liquid, containing the tocopherols, 20 cc. of the glacial acetic acid reagent were added.

The other 5 cc. aliquot was prepared simultaneously by adding 5 cc. of SSE and 20 cc. of glacial acetic acid reagent. Since appreciable errors can be caused by changes in volume, all volatile solvents, especially the SSE, were allowed to come to room temperature. After the contents were mixed, both tubes were read in a Klett-Summerson photoelectric colorimeter at 30 minutes. This time was chosen because, when the instrument was calibrated, standard solutions of  $\alpha$ -tocopherol gave maximum readings at 30 minutes. The instrument was adjusted to read zero for a blank. Values were read from a calibration curve in the usual manner.

The sulfuric acid treatment was reintroduced because of the more satisfactory recoveries secured with it as compared with the use of adsorption alone. When synthetic *dl*- $\alpha$ -tocopherol dissolved in SSB was added to a muscle sample when this was first placed in the blender with the foaming solvent, and this and the original muscle were carried through the entire procedure, the figures for recovery of the added tocopherol (Table I) averaged only 74 per cent with adsorption alone and 91 per cent when the sulfuric acid treatment was also included. The presence in muscle extracts of some interfering substance removable by the acid treatment was suggested by the appearance of a brown, viscous material at the interface between the SSE and the sulfuric acid. Also, with extracts treated only by adsorption the readings decreased with time, whereas with extracts given both treatments the readings increased with time to a maximum at 30 minutes as is the case with standard solutions of tocopherol. For so extensive a manipulation, the recoveries after double treatment may be considered fairly satisfactory, although such recoveries may admittedly provide a false sense of precision. The complete analysis of muscle for its content of tocopherol requires about 6 hours from the time the muscle is removed from the animal to the final colorimetric reading.

Determined by this method, the tocopherol content of muscle from rats

on three different régimes is shown in Table II. The only data available for comparison are those of Mason (18) obtained by biological assay; the amounts found in muscles of animals on dog chow are in fair agreement with his. According to Mason's results 60 gm. of fresh tissue contained a mean fertility dose. If this is accepted to be 1 mg. of  $\alpha$ -tocopherol (19), the  $\alpha$ -tocopherol content should be 16.7 mg. per kilo. This comparison suffers from the large variation (0.56 to 1.71 mg.) in mean fertility dose upon which this calculation depends. From the average figure herein obtained, 11.78 mg. per kilo, the mean fertility dose (contained in 60 gm. of fresh muscle) becomes 0.706 mg. The comparison also suffers from the

TABLE I  
*Recovery of  $\alpha$ -Tocopherol Added to Muscle Tissue*

Method of purification	Tocopherol content of muscle sample	Tocopherol added	Tocopherol found in muscle with tocopherol added	Recovery $\frac{(c-a)}{b} \times 100$
	(a)	(b)	(c)	
	$\gamma$	$\gamma$	$\gamma$	per cent
Adsorption only	44.6	210.0	216.0	81.6
	73.6	270.0	288.0	79.4
	188.0	220.0	348.0	72.7
	203.8	208.0	342.0	66.4
	80.6	210.0	231.0	71.6
Average .....				74.3
Adsorption and Parker-McFarlane	61.4	210.0	270.0	99.3
	121.4	220.0	324.0	92.0
	119.7	208.0	312.0	92.4
	22.0	210.0	192.0	80.9
Average .....				91.1

fact that  $\alpha$ -tocopherol is more effective biologically than the  $\beta$  and  $\gamma$  varieties and the dietary sources cannot be expected to contain only  $\alpha$ -tocopherol.

Deficient animals (except severely deficient males) differed but slightly from normal animals on a dog chow ration in respect to the amount of their muscle tocopherols. Mason (18) considers dog chow a suboptimal supply, containing only about 4 times the minimum daily requirement. Older female animals on a vitamin E-deficient ration appear to retain a higher concentration of tocopherols in their muscle tissue than do old males. This apparent sex difference in requirement has been discussed by Mason (20). In comparison with females, the requirements of male

animals may continuously be more insistent; perhaps female animals, in the absence of reproductive demands, can husband their stores more economically because of their lower energy exchange. The onset of external symptoms of deficiency in males (awkward gait and general senility) was rapid. No biological assays of muscle from vitamin E-deficient animals are available for comparison.

In agreement with the results of Mason (18) the tocopherol content of rat muscle was increased by feeding a diet rich in tocopherol. The dietary enrichment was much less extensive, both in amount and duration, than Mason's, and the results are less striking but nevertheless definite. The process of muscle enrichment is apparently slow.

TABLE II  
Average Tocopherol Content of Rat Muscle in Mg. per Kilo

No. of rats and diet	Extracts treated by		Diet	Extracts treated by	
	Adsorption only	Adsorption and Parker-McFarlane procedure		Adsorption only	Adsorption and Parker-McFarlane procedure
6 ♀, normal, on dog chow	10.53 (7.24-16.34)	11.78 (9.60-13.62)	Males, excess vitamin E	16.48 15.42*	17.60 17.63*
4 ♂, 5-6 mos., vitamin E-deficient	13.00 (10.15-16.40)	9.35 (6.46-11.00)		11.67* 20.70*	21.00* 19.95*
6 ♀, over 8 mos., vitamin E-deficient	11.22 (8.41-12.25)	9.51 (7.37-12.90)		21.95* 22.40*	19.80* 23.00*
4 ♂, 8-10 mos., vitamin E-deficient	4.94 (4.16- 5.89)	7.51 (6.21- 8.53)		21.80†	21.00†
			Average ...	18.63	20.00

\* These animals show the effect of 75 mg. of  $\alpha$ -tocopherol per week over a period of 1 to 5 weeks, respectively.

† Received a diet containing 3 mg. of  $\alpha$ -tocopherol acetate per kilo, over a 2 month period.

The only other chemical determinations of the vitamin E content of muscle are those made on the cow and the horse (21). Extracts were prepared by saponification, and the potentiometric and colorimetric methods both indicated about 5 mg. per kilo. Those figures are considerably lower than these given here for normal rat muscle, perhaps due to species differences, perhaps to the destructive action of saponification which is here avoided.

The recent observation (22) that  $\alpha$ -tocopherol phosphate has an effect on vitamin E-deficient muscle slices, *in vitro*, which tocopherol does not have, raises the question whether tocopherol may be present in tissues in a combined form not responsive to oxidation by ferric chloride. This would

appear unlikely in view of the consistently lower figures that are obtained on saponified preparations, presumably due to destruction. Such a situation might, however, help to explain the apparent similarity in tocopherol content of muscles from normal and from vitamin E-deficient rats. If the physiological action of tocopherol involves alterations which are in part reversible (23), the functional relations might prove to be of greater consequence than the actual amounts present. The physiological and chemical aspects of this problem are receiving further study. In any case, until a practical method is developed for the separate determination of the three tocopherols, the use of this adaptation of the Emmerie and Engel method must be confined to experimental animals on synthetic diets containing  $\alpha$ -tocopherol.

#### SUMMARY

A chemical procedure is described for the determination of tocopherols in animal tissues, which makes use of the iron- $\alpha, \alpha'$ -bipyridine color reaction. The procedure avoids the destructive action of saponification and includes removal of interfering substances, especially of cholesterol, by treatment with sulfuric acid and by adsorption.

As determined by this method, the amounts of tocopherol found in the muscles of normal and of vitamin E-deficient rats (except severely deficient males) were surprisingly alike, averaging 11.8 and 9.4 mg. per kilo, respectively. The muscle of old severely deficient males contained slightly less tocopherol, 7.5 mg. per kilo. By oral administration of  $\alpha$ -tocopherol the concentration of this vitamin in rat muscle was slowly increased to as much as 20.0 mg. per kilo.

The significance of these findings and the limitations of the method are briefly discussed.

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# THE COMPARATIVE AVAILABILITIES OF *d*(+)- AND *l*(-)-HISTIDINE FOR THE PRODUCTION OF LIVER GLYCOGEN

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Some years ago Dakin observed that the administration of histidine produced too little "extra glucose" in the phlorhizinized dog to justify considering it a glucose former (1). The consequent tendency to classify this amino acid as non-glycogenic has since been challenged by the discovery that the liver contains an enzyme, histidase, capable of converting it into *l*(+)-glutamic acid (2, 3), whose glycogenic character is not questioned (4, 5). This and the observation that *d*(+)-histidine is not attacked by histidase (2, 3) led us to test comparatively the capacities of *l*(-)- and *d*(+)-histidine to promote deposition of liver glycogen in the fasted rat. A similar study of *l*(-)-histidine has recently been published by Remmert and Butts (6) who found that rats fed this isomer form liver glycogen and show a decreased output of acetone bodies, both after some delay. Our data on the formation of liver glycogen from *l*(-)-histidine differ somewhat from theirs.

## EXPERIMENTAL

The *l*(-)-histidine used in our studies was isolated as the *l*(+)-histidine monohydrochloride, from spray-dried blood according to the method of Cox, King, and Berg (7), and from blood corpuscle paste by the procedure of Gilson (8). The *d*(+)-histidine was prepared as the *d*(-)-histidine monohydrochloride, essentially as outlined elsewhere (9). Optical rotations read with an electric sodium lamp on 2 per cent solutions of the monohydrochlorides in water containing 1 equivalent of hydrochloric acid gave  $[\alpha]_D^{25}$  values of  $+8.50^\circ$  and  $-8.50^\circ$ , respectively (9, 10). Both isomers melted at  $253-255^\circ$  (cf. (10)). The *l*(+)-glutamic acid hydrochloride used for comparative study was the highest purity Eastman Kodak Company product (m.p.  $209-210^\circ$ ).

Two series of tests were conducted, the first in midwinter, the second in late spring. For each, rats of both sexes were obtained from Sprague-Dawley, Inc., and maintained on a stock diet of commercial dog chow until ready for use. The animals in the first series were fasted for 24 hours before the feedings were begun and those in the second, for 48 hours. Their weights at the end of the fast ranged between 94 and 150 gm. During

the fasting period and the 4, 8, or 12 hours which followed the initial feeding, the rats were housed in individual cages provided with raised screen floors and were given continuous access to water and regenerated cellulose. Before the feedings were begun, the pans under the cages of the rats fed histidine were cleaned and fitted with fresh filter\* paper to absorb the urine voided during the metabolism period. The histidine and the glutamic acid were fed by stomach tube in the form of suspensions prepared by dissolving the hydrochloride of the amino acid in an aqueous suspension of gum tragacanth, then adding sodium hydroxide in an amount calculated to react with 0.9 of the hydrochloride, and finally exactly enough sodium bicarbonate to complete the conversion to sodium chloride. Control rats were fed similar gum tragacanth suspensions containing sodium chloride in like amount. The animals in the 4 hour groups received only the initial dose, those in the 8 hour groups were fed again after 4 hours, and those in the 12 hour groups after 4 and after 8 hours. The volumes of the dosages were 2 cc. or less. Except when odd numbers of rats were employed, each of the several groups was divided evenly as to sex.

The rats were killed by a blow on the head. The livers were removed for analysis in their entirety by an adaptation of the Good, Kramer, and Somogyi procedure (11). The gastrointestinal tracts of the animals fed *l*(-)- and *d*(+)-histidine were prepared for analysis for unabsorbed histidine essentially as described by Doty and Eaton (12). Feces eliminated after the initial feeding were added to the contents of the tracts. Aliquots of the deproteinized extracts were analyzed for histidine by the highly specific Pulfrich photometer method of Kapeller-Alder (13), essentially as modified by Conrad and Berg (9). Imidazole, 4-imidazole-formaldehyde, and  $\beta$ -4-imidazolelactic acid did not respond. Preliminary tests showed that the method usually accounted for over 95 per cent of the varied amounts of histidine added to aliquots of gastrointestinal washings prior to deproteinization. No histidine was found in the intestinal washings of any of the rats fed the control suspensions. The urine was obtained by washing the cage bottoms, filter paper, and pans with hot distilled water. The washings were decolorized with charcoal and analyzed for histidine, as above. The second of the two series of tests was completed in about a month, an interval shorter than for the first. Also in the second, special care was taken to include animals from several of the groups in each of the smaller lots fed and analyzed in a single day.

Average absorption coefficients (rates of absorption in mg. per 100 gm. of rat per hour) for each of the 4, 8, and 12 hour groups fed histidine are recorded in Table I. Variations among individual rats ranged from  $\pm 3$  mg. from the average in some groups to  $\pm 15$  mg. in others. The rates of absorption for the 4 hour groups agree rather well, as do also the rates

for all but the first of the 8 and 12 hour tests. It is possible that some incipient gastrointestinal disturbance, such as we have observed in acute form after longer periods in which large amounts of lysine were fed (14), may have retarded absorption in these instances. Remmert and Butts found even smaller amounts of *l*(-)-histidine toxic (6). The absorption coefficients for *l*(+)-histidine monohydrochloride, determined in 1 and 2 hour periods by Doty and Eaton (12), are 78.7 and 57.4 mg., respectively,

TABLE I  
*Absorption, Excretion, and Retention of Histidine by Rats Fed l(-)- or d(+)-Histidine after a Fast of 24 or 48 Hours*

Series No.*	No. of rats	Average weight	Period after initial feeding	Histidine fed†		Average rate of absorption‡	Average histidine	
				Isomeric form			Excretion	Retention
		gm.	hrs.		mg.	mg. per 100 gm. per hr.	mg.	mg.
I	10	108	4	<i>l</i> (-)	596	77.0	45	287
	10	121	8	"	1017	48.8	49	421
	10	114	12	"	1375	44.7	88	522
	6	106	4	<i>d</i> (+)	500	85.5	52	311
	6	105	8	"	750	67.7	97	469
	6	107	12	"	1000	64.6	161	663
II	6	105	4	<i>l</i> (-)	500	78.2		332
	6	104	8	"	750	69.2	45	533
	5	111	12	"	1000	59.0	53	719
	3	99	4	<i>d</i> (+)	500	72.5	46	240
	3	107	8	"	750	62.2	182	342
	3	109	12	"	1000	60.2	210	570

\* Rats in Series I were fasted for 24 hours before the initial feeding, rats in Series II for 48 hours.

† Rats in the 8 and 12 hour groups were fed initially the same amount of histidine as the rats in the corresponding 4 hour group. The difference between this and the total for 8 hours represents the amount fed at 4 hours, between the 12 hour and 8 hour totals, the amount fed at 8 hours.

‡ No histidine was found in any of the gastrointestinal tracts of the thirty-seven rats fed all of the ingredients of the suspension except histidine.

when expressed in terms of *l*(-)-histidine. In our studies conversion of the monohydrochloride to the free amino acid and sodium chloride may have been responsible for the relatively higher average coefficients of 78.3, 66.4, and 61.3 mg. obtained in the four 4 hour, three 8 hour, and three 12 hour periods. Remmert and Butts report coefficients of 51.4 mg. in a 4 hour period and 39.8 in an 8 hour period, but give no details (6).

Excretion of histidine was not detected in the rats fed 500 mg. of *l*(-)-histidine, but with a single exception it occurred in all of the other animals.



Variations within each group were wide. The *d*(+) form of histidine was the more readily excreted. Since the method of analysis was highly specific for histidine, the data afford no information as to total imidazoles eliminated. The estimates of retentions therefore serve only to indicate maximum quantities of histidine available for metabolism during the

TABLE II  
*Deposition of Liver Glycogen in Rats Fed l(-)-Histidine, d(+)-Histidine, or l(+)-Glutamic Acid after a Fast of 24 or 48 Hours*

Suspension fed	Period after initial feeding	Content of liver glycogen in groups fasted 24 hrs. (Series I)			Content of liver glycogen in groups fasted 48 hrs. (Series II)		
		Minimum	Maximum	Average	Minimum	Maximum	Average
	hrs.	per cent	per cent	per cent	per cent	per cent	per cent
<i>l</i> (-)-Histidine*	4	0.22	0.57	0.36	0.77	1.09	0.90
	8	0.22	0.85	0.47	0.87	1.67	1.09
	12	0.14	0.98	0.54	0.66	2.59	1.44
<i>d</i> (+)-Histidine*	4	0.04	0.28	0.19	0.23	0.59	0.46
	8	0.08	0.47	0.23	0.57	1.36	0.96
	12	0.05	0.12	0.09	1.03	1.16	1.09
<i>l</i> (+)-Glutamic acid† (274 mg.)	4				0.32	1.45	0.97
"    "    (400 " )	8				0.99	1.72	1.52
"    "    (495 " )	12				0.96	2.28	1.83
Control‡	4	0.08	0.39	0.19	0.22	0.49	0.36
	8	0.06	0.28	0.13	0.59	0.78	0.66
	12	0.04	0.28	0.11	0.51	1.11	0.73

\* Consult Table I for the number of rats and the average weights of the rats fed histidine, the amount of histidine fed, and estimates of the amount retained.

† The glutamic acid fed each of the six rats in each group is indicated in the parentheses opposite the period after initial feeding. The amount was equivalent to the average *l*(-)-histidine retained in a corresponding period by rats in Series I (see Table I). The average rat weights in the groups were 107, 107, and 118 gm., respectively.

‡ The number of rats and average weights of the rats in the 4, 8, and 12 hour control groups of Series I were 5, 7, 7, and 108, 118, and 121 gm., respectively. In Series II each control group contained six animals; in the three groups the average weights were respectively 105, 111, and 110 gm.

period. The amounts retained by the individuals in each group varied widely.

Table II presents the results of the analyses for liver glycogen. Though the responses in the two series of tests differed quantitatively, the data from both show definitely that *l*(-)-histidine promotes the deposition of liver glycogen. Statistical evaluation supports this conclusion. In each instance comparison of an experimental group with its control group

gave a value above 3 for the ratio of the mean difference to the probable error of the mean difference. The lag in glycogen production from *l*(-)-histidine indicated by Remmert and Butts (6) was not observed.

The response to *d*(+)-histidine was strikingly different in both series. Statistical comparisons with control groups suggest that only the results obtained in the 12 hour period of the second series of tests are definitely positive. It seems fair to conclude that *d*(+)-histidine is much less readily converted than *l*(-)-histidine to liver glycogen.

The *l*(+)-glutamic acid was fed in amounts designed to approximate the maximum which could have been produced from histidine had all of the *l*(-)-histidine retained been converted by histidase to *l*(+)-glutamic acid. The quantities fed were based on the average retentions of *l*(-)-histidine in corresponding periods in the first series of tests. Although the average retentions in the simultaneous studies on *l*(-)-histidine proved to be 16, 27, and 38 per cent greater than thus anticipated, the data seem nevertheless to suggest that the rates of glycogen formation from *l*(+)-glutamic acid and *l*(-)-histidine were of about the same order. Evidence of this sort obviously does not prove that the *l*(-)-histidine was converted to liver glycogen via glutamic acid, but it does admit of such an interpretation. In harmony with that possibility, the less ready conversion of *d*(+)-histidine to liver glycogen may be explained by assuming that its obligatory conversion to *l*(-)-histidine is slow, a likelihood already suggested by its less efficient promotion of growth (9, 10).

#### SUMMARY

Under similar experimental conditions *l*(-)- and *d*(+)-histidine were absorbed by the rat at approximately equal rates. The rate of absorption per hour was greater in 4 hours than in 8 or 12. *d*(+)-Histidine was the more readily excreted.

Appreciable liver glycogen was formed when *l*(-)-histidine was fed. No lag was noted. The deposition when *d*(+)-histidine was fed was less marked and of doubtful statistical significance in all groups of tests except one of the two for a 12 hour period.

When *l*(+)-glutamic acid was fed in amounts approximately equivalent to the *l*(-)-histidine retained in similar periods, deposition of liver glycogen occurred at about the same rate. The data obtained in these studies admit of the possibility that *l*(+)-glutamic acid is formed as an intermediate in the conversion of histidine to liver glycogen.

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# SOLUBILITIES AND COMPOSITIONS OF THE PHOSPHO-12-TUNGSTATES OF THE DIAMINO ACIDS AND OF PROLINE, GLYCINE, AND TRYPTOPHANE

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Phosphotungstic acid (which will be designated as PTA) has long been used in separations of the amino acids. It was employed by Drechsel (1) in the discovery of lysine in casein, and by Hausmann (2), by Osborne, Leavenworth, and Brautlecht (3), and by Van Slyke (4-8) for quantitative precipitation of the diamino acids, arginine, lysine, and histidine, in protein hydrolysates. Cystine was found to be about half changed under the conditions of acid hydrolysis to a non-precipitable form (4) although the natural *l* form is precipitable with about the same completeness as histidine. The change was shown by Hoffman and Gortner (9) to be racemization, *dl*-cystine forming a more soluble phosphotungstate than that of *l*-cystine.

The monoamino acids also form crystalline phosphotungstates. Although many times more soluble than the PTA salts of the diamino acids, some of the monoamino phosphotungstates have been found useful for separations of individuals within the monoamino group (10). A nearly quantitative separation of alanine from valine has thus been obtained (11).

The phosphotungstates of the diamino acids, when precipitated under the conditions customarily employed with protein hydrolysates (2-8), *viz.* about 1 *N* HCl or H<sub>2</sub>SO<sub>4</sub> and room temperature, have solubilities sufficiently great to necessitate corrections for them in accurate quantitative work (4-6). The effects of varying temperature, acidity, and of coprecipitation of the different diamino acids together have, however, not heretofore been subjected to a systematic study. It appeared that such a study might increase the accuracy of solubility corrections, and perhaps also lead to discovery of conditions of precipitation which would make the phosphotungstate separation of the diamino acids from the monoamino group more sharp and certain.

## Materials Used

*Phosphotungstic Acid*—The PTA used was the 12-acid, and had the composition H<sub>3</sub>PO<sub>4</sub>·12WO<sub>3</sub>·7H<sub>2</sub>O. Wu (12) has given a method for preparing the 12-acid from tungstic and phosphoric acids. We have, however, made our preparations by applying Winterstein's (13) purification method to commercial PTA; the acid is dissolved in water and extracted from the

water solution with ether; the heavy ethereal solution which settles below the water phase is then washed several times with water, and dried on a steam bath.

Phosphotungstic acid purified in this manner was used by Van Slyke in the original distribution method of protein analysis (4-6), and in all subsequent work from this laboratory with phosphotungstates. The purification seems to separate the commercial PTA into two definitely different fractions. The part which goes into the ether is removable therefrom only to a slight extent by subsequent washings of the ethereal solution with water, while the part which remains in the water after the first ether extraction is removable from the water to only a slight extent by additional extractions with ether. Different commercial preparations of PTA gave yields of the ether-soluble fraction varying from 30 to 80 per cent.

The PTA was analyzed by the following methods.

*Loss of  $H_2O$  on ignition* was determined by the technique of Toennies and Elliott (14), the sample being ignited over a Bunsen burner in a porcelain crucible for 10 minute periods until the weight becomes constant. We confirmed Toennies in finding a minimum weight reached sharply after one or two 10 minute ignitions. As found by Miolati and Pizzighelli (15), the ignition yields the product  $HPO_3 \cdot 12WO_3$ .

As shown by Table I, the analysis indicates a composition of  $H_3PO_4 \cdot 12WO_3 \cdot 7H_2O$  in the preparation dried on a steam bath. Portions dried at  $100^\circ$  *in vacuo* lost only 0.25 per cent of the weight, equivalent to 0.5 mole of  $H_2O$ . In the avidity with which water of crystallization is held, the PTA differs markedly from its salts with amino acids; at least some of these lose all their water in 30 minutes at  $100^\circ$  *in vacuo*.

*Equivalent Weight by Titration with Alkali*—As shown by Toennies and Elliott (14) a slight excess of alkali readily splits the PTA into tungstate and orthophosphate, and titration of the products to a pH of 9 forms  $Na_2WO_4$  and  $Na_2HPO_4$ , with neutralization of 26 equivalents of NaOH per mole of  $H_3PO_4 \cdot 12WO_3$ .

Samples of about 150 mg. of PTA were dissolved in enough 0.1 N NaOH to give a permanent red color with phenolphthalein. Then 2 or 3 cc. excess of the NaOH was added, and the solution was heated till it began to boil in order to split the phosphate from the tungstate. To the hot solution 0.1 N sulfuric acid was then added from another burette until a few drops more than enough to decolorize the phenolphthalein were present. The solution was then boiled for a minute to expel any  $CO_2$ , and was titrated with 0.1 N NaOH to the color of a standard solution containing the same indicator and 100 mg. of  $Na_2HPO_4$ .

*Tungstic Acid*—The tungsten was precipitated with tannin by the method of Barber (16), was ignited overnight in a muffle at  $600^\circ$ , and weighed as  $WO_3$ .

*Phosphoric Acid*—In samples of about 2.5 gm. the  $PO_4$  was determined as directed by Wu (12), the  $PO_4$  being precipitated with magnesia mixture, and the precipitate ignited to render adsorbed  $WO_3$  insoluble. The phosphate is extracted from the residue with dilute HCl and reprecipitated with magnesia mixture. We followed Treadwell-Hall's (17) procedure of washing the precipitate with alcohol and ether, drying 20 minutes *in vacuo*, and weighing as the crystalline  $MgNH_4PO_4 \cdot 6H_2O$ .

The results of the analyses are shown in Table I.

*Amyl Alcohol*—The amyl alcohol used for extracting phosphotungstic

acid from the supernatant solutions was purified by shaking it with 1 N HCl to remove nitrogenous bases, and was then distilled at 15 to 20 mm. pressure. The middle half of the distillate was used. Even thus purified the amyl alcohol added a small but definite amount to the blanks of the amino nitrogen determination. For this reason blanks were always determined on solutions treated in the same manner as the supernatant solutions from the phosphotungstates: A portion of aqueous HCl equal in volume and concentration to the supernatant solution was treated with amyl alcohol and ether, concentrated to dryness, and the residue was taken up in water and analyzed in the same way as the supernatant solution.

*Amino Acids*—The amino acids used were all subjected to elementary analysis, and were found free from impurities sufficient to affect the analytical figures. All (except the glycine) were the natural optically active

TABLE I  
*Analysis of Phosphotungstic Acid Used*

Analysis	Found	Calculated for $\text{H}_3\text{PO}_4 \cdot 12\text{WO}_3 \cdot 7\text{H}_2\text{O}$
	<i>per cent</i>	<i>per cent</i>
Loss on ignition to $\text{HPO}_3$ and $\text{WO}_3$ .	4.78	4.79
	4.96	
	4.72	
	4.72	
Equivalent weight by titration to $\text{Na}_2\text{HPO}_4$ and $\text{Na}_2\text{WO}_4$	114.6	115.6
	114.2	
	114.8	
$\text{WO}_3$ content	92.8	92.7
	93.2	
Phosphorus, calculated as P	1.055	1.031
	1.049	
	1.036	

isomers, and gave rotations agreeing with those in the literature. Tryptophane, glycine, and cystine were analyzed and used as the free amino acids, arginine as the monochloride, and histidine and lysine as the dichlorides. These preparations all showed in their phosphotungstates the solubility behavior of pure substances.

The proline was prepared from analyzed proline picrate from hydrolyzed gelatin. Although it was entirely free of amino nitrogen determinable with nitrous acid, and gave correct figures on elementary analysis, it contained impurity enough to make the solubility of its phosphotungstate measured by precipitation higher than that by resolution. The impurity was removed by recrystallization of the phosphotungstate. From the fact that the impurity was determinable by the ninhydrin- $\text{CO}_2$  method but not by nitrous acid, it is probable that it was hydroxyproline.

## TECHNIQUE OF SOLUBILITY MEASUREMENTS

*Approach of Solubility Equilibrium from Both Sides*—The solubility data on amino acid phosphotungstates in the literature have been based on precipitation experiments: the amino acids and phosphotungstic acid have been mixed in solution, and, after a longer or shorter time allowed for precipitation to become presumably complete, the nitrogen or amino acid content of the supernatant liquid has been determined. Such experiments may give too high results from two sources: (1) The time allowed for precipitation may not be long enough for complete attainment of equilibrium. (2) Any nitrogenous impurity in the amino acid preparation used would probably remain in the supernatant solution. In the cases of the diamino acids, with slightly soluble phosphotungstates, even slight amounts of such impurities can greatly increase the apparent solubilities.

To detect such errors we have approached the solubility equilibrium from both sides. The solubilities were first determined by *precipitation*. Then the precipitates, in some cases after recrystallization, were shaken in fresh solutions of phosphotungstic acid, and the solubility was determined by *resolution*, by measuring the amount of amino acid redissolved.

*Solubility by Precipitation*—Each amino acid was dissolved in aqueous HCl of desired concentration, and weighed amounts of phosphotungstic acid were added. The mixture was heated until solution was complete, and was then cooled to the temperature of the experiment. It was then let stand long enough for the amino acid concentration in the supernatant solution to reach a constant minimum.

The volumes and concentrations of the precipitating amino acid solutions were varied with the magnitude of the solubilities. In the cases of lysine, histidine, arginine, and cystine about 200 mg. of each amino acid or its hydrochloride were dissolved in 200 cc. of HCl solution of definite normality. Glycine, proline, and tryptophane form much more soluble phosphotungstates; so that more concentrated solutions were required to exceed the solubilities, and less supernatant volume was needed for analyses. Of these amino acids 10 to 50 mg. per cc. of solvent solution were used, and volumes of 10 to 20 cc. sufficed for each experiment.

The amount of phosphotungstic acid added was uniformly calculated to provide enough to combine with the amino acid in the ratio of 1 mole of phosphotungstic acid to 3 moles of monoamino acid or 1.5 moles of diamino acid (see Table II), and to leave a desired excess of free phosphotungstic acid in solution. Except in experiments in which the free phosphotungstic acid was varied, the excess used was calculated to leave 50 gm. (0.0166 mole) per liter in the supernatant solution.

The time required for complete precipitation was found to vary inversely as the solubility. At room temperature arginine and lysine, which form

the least soluble phosphotungstates, reach maximum precipitation in a few hours, histidine and *l*-cystine 48 hours, glycine and proline 72 to 96 hours.

*Solubilities by Resolution*—The precipitates obtained in the precipitation experiments were freed as completely as possible of supernatant solution by suction on hardened filter paper, and were resuspended in fresh solutions of HCl and PTA. In saturations at 22° the mixtures were shaken mechan-

TABLE II  
*Compositions of Amino Acid Phosphotungstates*

Group	Amino acid	Method of drying phosphotungstate*	Composition of phosphotungstate			Molecular ratios		Molecular formula approximated†
			PTA as anhydrous $\text{H}_2\text{PO}_4 \cdot 12\text{WO}_3$	Amino acid	$\text{H}_2\text{O}$	Amino acid PTA	$\text{H}_2\text{O}$ PTA	
			(a)	(b)	(c) = $\frac{100}{(a+b)}$			
			per cent	per cent	per cent			
Diamino acids	Arginine	Des. + air	89.64	8.21	2.15	1.51	3.8	$\text{A}_3\text{P}_2 \cdot 8\text{H}_2\text{O}$
	Histidine	" + "	90.65	7.64	1.71	1.47	3.0	$\text{A}_2\text{P}_2 \cdot 6\text{H}_2\text{O}$
	" ‡	Air	89.24	7.32	3.44	1.52	6.2	$\text{A}_2\text{P}_2 \cdot 12\text{H}_2\text{O}$
	Lysine	Des. + air	90.51	6.75	2.74	1.47	4.8	$\text{A}_2\text{P}_2 \cdot 10\text{H}_2\text{O}$
	Cystine	"	88.45	8.16	3.39	1.11	6.1	$\text{AP} \cdot 6\text{H}_2\text{O}$
Mono-amino acids	Glycine	"	89.99	7.10	2.91	3.03	5.2	$\text{A}_3\text{P} \cdot 5\text{H}_2\text{O}$
	Proline	" + air	89.06	9.54	1.40	2.68	2.5	$\text{A}_2\text{P} \cdot 2.5\text{H}_2\text{O}$
	Tryptophane	"	80.70	16.81	2.49	2.94	4.9	$\text{A}_3\text{P} \cdot 10\text{H}_2\text{O}$

\* Des. signifies dried in a desiccator to constant weight, at 20 to 30 mm. pressure over calcium chloride, at room temperature. Des. + air signifies dried as above, then permitted to take up water from room air till constant. The amount taken up was 1.0 to 1.3 per cent. Air signifies that the wet crystals were left 48 hours spread thin on a porous plate, protected from dust, but in free contact with room air.

† In the formulae, A represents 1 molecule of amino acid and P 1 molecule of  $\text{H}_2\text{PO}_4 \cdot 12\text{WO}_3$ .

‡ This histidine phosphotungstate was recrystallized from 0.2 N hydrochloric acid.

ically for 3 to 5 hours. In experiments at 4° and 38° the mixtures were let stand for 24 hours, with occasional shaking by hand. Control experiments showed that these conditions sufficed to approach complete saturation. The volumes used were the same as for solubility by precipitation, varying inversely as the solubilities.

*Recrystallization of Phosphotungstates*—When solubilities by resolution as described above were lower than solubilities by precipitation, indicating



possible impurities in the amino acid preparations used, the phosphotungstates were recrystallized and the solubilities were redetermined by both methods. For the recrystallization the phosphotungstates were redissolved in hot water, and were reprecipitated with addition of phosphotungstic and hydrochloric acids to give the desired concentrations in the supernatant solutions.

Solubility determination proved to be a more sensitive means than elementary analysis for detecting impurities. The first preparation of proline picrate used showed no impurities detectable by either analysis or rotation, but the solubility behavior revealed impurities in the form of other amino acids. A preparation of analytically pure lysine picrate was similarly found by solubility to be impure, and was replaced by a preparation of lysine dichloride which gave identical solubilities by both methods.

*Analyses of Supernatant Solutions*—From the supernatant solutions of the difficultly soluble phosphotungstates, *viz.* those of lysine, histidine, and arginine, and of cystine at 0°, sufficient volumes were concentrated to 15 cc., so that a 5 cc. portion of the concentrated solution used for amino nitrogen<sup>1</sup>

<sup>1</sup> *Analytical Peculiarities of Certain Amino Acids*—In analyses of the amino acids the following peculiarities are to be noted:

Neither lysine nor tryptophane gives quantitative nitrogen values by Kjeldahl analysis. The yields of ammonia are variable, but usually about 90 per cent of the theoretical. Trial of practically all the oxidizing and catalytic agents recommended in the literature for use in Kjeldahl digestion has not, in our hands, improved the results.

Arginine and tryptophane give theoretical values for  $\alpha$ -amino nitrogen by the nitrous acid method with the regular reagents (18, 19) in the reaction time used for  $\alpha$ -amino groups (4 minutes at 20°, 3 at 25°), but if the reaction is prolonged beyond this period the results become high. For each additional " $\alpha$  period" of 4 minutes at 20°, arginine evolves 0.4 per cent of an extra  $N_2$  molecule, and tryptophane 1.2 per cent. The secondary reaction of arginine, due doubtless to the  $NH_2$  of the guanidine group, has been noted by Hunter (20).

Glycine, which gives about 108 per cent of the theoretical  $N_2$  by Van Slyke's original nitrous acid method (18), gives 102 to 103 per cent when the later manometric apparatus (19) is used. The mercury in the manometric chamber apparently retards the side reaction that causes the high results encountered when the former apparatus, without mercury, is used. This behavior has been discussed by Kendrick and Hanke (21) who found that adding KI lowered the results to theoretical.

Cystine, on the other hand, which likewise by the original nitrous acid method (18), in apparatus without mercury, gave 108 per cent of theoretical  $N_2$  yields, in the presence of the mercury of the manometric method (19), about 130 per cent. We have confirmed Kendrick and Hanke (21), however, to the effect that adding KI to the nitrite solution used for the manometric method entirely prevents this error in cystine analyses.

Potassium iodide cannot be used in amino nitrogen determinations in which tryptophane forms a significant fraction. As Kendrick and Hanke (21) have shown, KI so affects the reaction of nitrous acid with tryptophane that only 40 to 60 per cent of theoretical  $N_2$  is obtained from the tryptophane  $NH_2$  group.

determination would contain enough  $\text{NH}_2$  nitrogen (0.1 to 0.5 mg.) to permit analysis with an accuracy of the order of 1 part in 200 by the manometric nitrous acid method (19). For the solubility determinations at the different temperatures the following volumes of supernatant solutions of these amino acids were concentrated to 15 cc.; at 0° 300 cc., at 22° 150 cc., at 38° 100 cc. Before the solutions were concentrated the phosphotungstic acid was removed by extraction with amyl alcohol and ether in the presence of 1 N HCl (or stronger, in cases in which the precipitations had been carried out in 2 or 3 N HCl), by the technique formerly described (5). The free HCl in the solution was then distilled off by concentration to dryness *in vacuo* in a double necked distilling flask. The residue was taken up in a few cc. of water, with the addition of a few drops of 1 N NaOH to neutralize and clear the solution, and was washed into a 15 cc. volumetric flask. Until the alkali was added there was usually a slight turbidity, due perhaps to precipitation of amino acid by traces of unextracted phosphotungstic acid.

In some of the experiments with histidine, the histidine contents of the supernatant solutions were determined photometrically by the method of Jorpes (22), with the Zeiss step-photometer. This method could be applied to such dilute solutions that neither concentrating the supernatant solutions nor removing the phosphotungstic acid was necessary. The error of the colorimetric procedure is greater than that of the manometric, but appears to be within  $\pm 2$  per cent. In preparing the control curves it was found essential to have the reagents act on the standard histidine solutions at the same temperature used in the analyses of the supernatant solutions.

In the supernatant solutions of tryptophane and of cystine at 22° and 38° the amino nitrogen concentrations of the supernatant solutions were great enough to permit analyses by the manometric nitrous acid method (19) without preliminary concentration. Portions of 3 cc. of tryptophane supernatant solution, or of 10 cc. of cystine supernatant, were pipetted directly into the manometric apparatus and were neutralized there by addition of 18 N NaOH. In the amino nitrogen determinations on cystine supernatant solutions KI was added to the nitrite reagent, as recommended by Kendrick and Hanke (21).

In the cases of glycine and proline the amino acid content of the filtrate was determined by analyzing 0.5 or 1.0 cc. for amino acid carboxyl  $\text{CO}_2$  by the ninhydrin- $\text{CO}_2$  method (23). The sample solution was placed in a reaction vessel (23), and 1 or 2 N NaOH was added until the pH, indicated by brom-cresol green, approximated 4.7. 50 mg. of citrate buffer of pH 4.7 were then added, preformed  $\text{CO}_2$  was boiled off, and the analysis carried out as previously described (23).

Measurements of hydroxylysine phosphotungstate solubilities observed when the amino acid was precipitated together with varying proportions of lysine have already been reported by Van Slyke, Hiller, and MacFadyen (24), who determined the hydroxylysine in the supernatant solutions by means of their periodate-ammonia method for the  $-\text{CH}(\text{NH}_2) \cdot \text{CH}(\text{OH})-$  group.

### *Composition of Amino Acid Phosphotungstates*

Most authors have found the ratio of 3 moles of monoamino acid or 1.5 moles of diamino acid per mole of PTA in the precipitates (16, 25-27). The analyses have not in all cases been complete, however, being based often only on nitrogen determinations, and it has not been certain to what extent the compositions of the precipitates depended on the conditions of their formation. Toennies (14) found that in the case of cystine phosphotungstate, instead of the expected ratio of 1.5, ratios varying from 1 to 1.75 could be obtained according to the conditions of precipitation.

We have prepared the phosphotungstates by precipitation from solutions of the concentrations, indicated on p. 140, used in solubility determinations. Enough PTA was used in each case to combine with the amino acid in the ratio of 1:3 for the monoamino acids, or 1:1.5 for the diamino, and leave approximately 50 mg. of free PTA per cc. in the supernatant solution. The precipitates were washed with cold water on hardened filter papers, and were dried for analysis in the ways indicated in Table II. We did not attempt to define with precision the conditions for drying to constant content of water of crystallization, since the water of crystallization, to judge from the literature, is not definite under ordinary conditions of preparation, and the object of the analyses was to obtain the ratio of amino acid to PTA.

*Amino Acid Content*—For amino nitrogen determinations, samples were weighed into volumetric flasks in such amounts that when dissolved and made up to volume 5 cc. portions of the solution would contain 70 to 80 mg. of phosphotungstate in the cases of arginine and histidine, 30 to 40 mg. in the cases of lysine, cystine, glycine, and tryptophane. The phosphotungstates were dissolved with the aid of just enough 1 N sodium hydroxide to make them alkaline to alizarin and give clear solutions. Portions of 5 cc. were used for determination of amino nitrogen by the manometric nitrous acid method (19). For lysine the nitrous acid reaction was prolonged to 5 times the interval used for  $\alpha$ -amino groups, in order to obtain complete reaction of the  $\omega$ - $\text{NH}_2$ . For the other amino acids the usual reaction time of the  $\alpha$ -amino groups was used; e.g., 4 minutes at 20° ((19) p. 440). For the analysis of cystine, KI was added to the nitrite reagent in the proportions recommended by Kendrick and Hanke (21).<sup>1</sup>

Proline phosphotungstate was analyzed by the ninhydrin- $\text{CO}_2$  manometric method of Van Slyke, Dillon, MacFadyen, and Hamilton (23). Of the phosphotungstate 40 to 50 mg. were dissolved in 2 cc. of water and a few drops of 1 N NaOH were added to

bring the pH to about 4.7, as indicated by the color given to brom-cresol green. Citrate buffer of pH 4.7 was then added and the analysis was carried through at that pH.

**Phosphotungstic Acid Content**—The ignition technique of Toennies and Elliott (14) was used, in which the residue is weighed as  $\text{HPO}_4 \cdot 12\text{WO}_3$ . Samples of 150 to 200 mg. were ignited in an open porcelain crucible for a few minutes until the carbon was burned off. The crucible was then covered and subjected to the full heat of a Bunsen burner for 20 minutes, which was found sufficient to give a maximum weight loss. Duplicate analyses checked within 0.3 per cent. The values for  $\text{H}_2\text{PO}_4 \cdot 12\text{WO}_3$  were calculated by multiplying the ignition residue values by the theoretical factor, 2881/2863, or 1.0063.

**Water Content**—The water of crystallization was calculated in percentage terms as

$$\text{H}_2\text{O} = 100 - (\text{amino acid content}) - (\text{H}_2\text{PO}_4 \cdot 12\text{WO}_3)$$

This indirect procedure for estimating the water suffers from addition of the errors of the amino acid and the ignition residue determinations, and the results cannot be considered accurate by more than 0.5 mole of the  $\text{H}_2\text{O}$  per mole of PTA. However, we have used the method because direct estimation of the water by drying methods suffers from uncertainty concerning the readiness with which the water is driven off from the different phosphotungstates. Thus Drummond (25) obtained what he apparently considered were satisfactory water values in several phosphotungstates by drying at 105–110°, while Toennies (14) found that preparations of cystine phosphotungstate dried at 110° might still contain as much as 3.5 per cent of water, when determined by ignition, and free phosphotungstic acid itself holds the composition  $\text{H}_2\text{PO}_4 \cdot 12\text{WO}_3 \cdot 7\text{H}_2\text{O}$  when dried either on the steam bath (present authors) or at 110° (Toennies (14); Miolati and Pizzighelli (15)). However, in the preparation of histidine and proline in Table II, we have checked the water contents by drying at 100° *in vacuo*, and found that the results agreed with those from ignition, as would be expected from Drummond (25).

The results in Table II indicate that under the conditions used in our precipitations, as under the somewhat different conditions used by previous authors (16, 25–27), the trivalent formula for the phosphotungstates is approximated, 3 moles of monoamino acid and 1.5 moles of diamino acid combining with 1 mole of the 12-phosphotungstic acid, except in the case of cystine. Our data confirm those of Toennies and Elliott (14), who found that cystine did not follow the trivalent formula.

#### SOLUBILITY RESULTS

**Non-Dependence of Solubility on Ratio of Substrate to Supernatant Solution**—That the concentration of a saturated solution in equilibrium with the solid phase of the solute is independent of the amount of solid phase present is a law of solubility so generally valid that one would not ordinarily consider its proof necessary for a special case. However, for the phosphotungstates of some of the amino acids Thimann (28) has reported that observed solubilities varied in the same direction as the amount of substrate present per unit of supernatant solution. Consequently, as a preliminary to our work, it became necessary to test this point.

Thimann's results were obtained by the precipitation method only, and the amount of PTA added per unit volume of precipitated solution was kept constant, while the amounts of amino acids added were varied. Solubilities measured by this procedure could be affected by two possible varying factors other than the amount of substrate. (1) Slight amounts of other amino acids contaminating the one added could increase the apparent solubility, measured from the nitrogen content of the supernatant solution, and the increase would be greater as the amount of added amino acid increased. (2) Addition of increasing amounts of amino acid, while the amount of PTA in the system was kept constant, as in Thimann's experiments, would decrease the concentration of free PTA left in the supernatant solution. With the decrease in concentration of free PTA the precipitation might be less complete at equilibrium. Here again the ap-

TABLE III

*Solubility of Arginine Phosphotungstate Determined with Varying Amounts of Substrate*

Solubilities at 22°, with supernatant solution containing 1 N HCl and approximately 16.6 mM of PTA (50 gm. per liter).

Arginine added	Precipitation (48 hrs.)		Resolution
	PTA added	Arginine in supernatant solution	Fresh 16.6 mM PTA, 1 N HCl, shaken with same ppts. Arginine in supernatant solution
<i>mM per l.</i>	<i>mM per l.*</i>	<i>mM per l.</i>	<i>mM per l.</i>
2.36	18.3	0.223	0.217
4.73	20.0	0.229	0.217
9.46	23.2	0.233	0.217
18.92	29.4	0.235	0.213

\* 1 mM of  $H_3PO_4 \cdot 12WO_3 \cdot 7H_2O = 3.007$  gm.

parent solubility would increase with increasing amounts of added amino acid.

We attempted to avoid these sources of error. The amino acids used were of purity tested both by elementary analysis and by the constancy of the solubilities of their phosphotungstates approached from both sides of equilibrium. The concentrations of free phosphotungstic acid in the supernatant solutions were kept approximately constant in the precipitation experiments by varying the amount of PTA added with the amount of amino acid, so that enough PTA would be present to combine with all the amino acid, in the proportions experimentally found (Table II), and in addition provide an excess to leave an approximately constant concentration of PTA in the supernatant solution.

The results in Tables III to VI with arginine, histidine, glycine, and with

the recrystallized phosphotungstate of proline show that the solubility in each case is independent of the amount of precipitated substrate. The "precipitation" results with unrecrystallized proline phosphotungstate in Table VI show how an impurity can cause an apparent increase in the solubility with increasing amounts of amino acid.

TABLE IV

*Solubility of Histidine Phosphotungstate Determined with Varying Amounts of Substrate*

Solubilities at 22°, with supernatant solution containing 1 N HCl and approximately 16.6 mm of PTA.

Histidine added <i>mm per l.</i>	Precipitation (48 hrs.)	
	PTA added <i>mm per l.</i>	Histidine in supernatant <i>mm per l.</i>
2.10	18.3	0.55
4.20	19.8	0.54
8.40	22.9	0.56
16.80	29.0	0.57

TABLE V

*Solubility of Glycine Phosphotungstate Determined with Varying Amounts of Substrate*

Solubilities at 22°, with supernatant solution containing 1 N HCl and approximately 16.6 mm of PTA.

Glycine added <i>mm per l.</i>	Precipitation (96 hrs.)		Resolution determinations with same ppts.
	PTA added <i>mm per l.</i>	Glycine in supernatant <i>mm per l.</i>	Glycine in supernatant <i>mm per l.</i>
89	46.3	39.0	37.0
133	60.9	37.0	37.2
266	105.3	36.6	37.0

### *Effects of Concentration of Free Phosphotungstic Acid*

In both Hausmann's (2) and Van Slyke's (4-6) methods for separating the hexone bases by PTA precipitation the concentration of PTA used was about 50 gm. (16.6 mm) per liter. The following experiments were performed to find whether deviations from this PTA concentration affect the solubilities of the precipitated phosphotungstates.

Histidine and arginine, in approximately 4 mm concentration each, were precipitated with PTA in the presence of 1 N HCl, and the precipitates were sucked free of gross adherent fluid on a filter. Portions representing approximately the amounts precipitated from 200 cc. of solution were

resuspended in that volume of solution containing HCl of 1 *N* concentration and PTA of varying concentration. The solutions were saturated by constant shaking for 3 hours at 22°, and the amounts of amino acid in the supernatant were determined by amino nitrogen estimation, as previously described. The results are given in Table VII.

It is apparent that for these bases some excess of free phosphotungstic acid is necessary to depress the solubility to its minimum, but that variations between 25 and 100 gm. of PTA per liter (8.3 to 33.3 mM) make little difference in the solubilities.

TABLE VI

*Solubility of Proline Phosphotungstate Determined with Varying Amounts of Substrate*

Solubilities at 22°, with supernatant solution containing 1 *N* HCl and approximately 16.6 mM of PTA. The variable results in the 3rd column illustrate the effect of an impurity in the proline preparation. The constancy of results in the last column evidences removal of the impurity by the recrystallization.

Proline added	Precipitation		Resolution determinations with same ppts. recrystallized
	PTA added	Proline in supernatant	Proline in supernatant
<i>mM per l.</i>	<i>mM per l.</i>	<i>mM per l.</i>	<i>mM per l.</i>
87	46	36.6	25.5
174	75	41.5	25.0
348	133	48.2	25.3

#### *Effect of Concentration of Free Hydrochloric Acid*

The solubilities were measured by the "resolution" technique, by shaking previously precipitated phosphotungstates for 3 to 5 hours at 22° with solutions containing PTA in 16.6 mM (50 gm. per liter) concentration, and varying concentrations of HCl. The amino acid concentrations in the supernatant solutions were determined by amino nitrogen or amino acid carboxyl determinations, as previously outlined.

The results in Table VIII indicate that 0.25 *N* HCl should be a better medium than 1 *N* HCl for the phosphotungstate separation of the diamino from the monoamino acids. The solubilities of the phosphotungstates of all the amino acids are lower in the more dilute HCl, but the percentage lowering is greatest in histidine and arginine, especially the latter, which is only 32 per cent as soluble in 0.25 *N* as in 1 *N* HCl. The phosphotungstates of the monoamino acids, glycine and proline, are relatively less affected; so that in the 0.25 *N* HCl a more complete separation appears attainable. For example, in 1 *N* HCl arginine phosphotungstate is 0.006 as soluble as glycine phosphotungstate; hence from a mixture of equimolar amounts one could precipitate 99.4 per cent of the arginine in a volume of solution which

would hold the glycine dissolved. In 0.25 N HCl, the arginine is only 0.002 as soluble as the glycine, and a 99.8 per cent separation should be possible.

In 2 and 3 N HCl the solubilities of all the phosphotungstates are also less than in 1 N. But the effect is not so markedly in favor of the diamino acids. Levene and Van Slyke (11) found that the solubility of alanine phosphotungstate also is low in the presence of 2 N acid.

TABLE VII

*Effect of Variation in Free PTA Concentration on Solubilities of Arginine and Histidine Phosphotungstates*

Temperature, 22°; HCl concentration, 1 N; solubility by resolution of previously precipitated phosphotungstates.

Concentration of PTA in supernatant solution		Solubility of arginine	Solubility of histidine
mm per l.	gm. per l.	mm per l.	mm per l.
0	0	0.666	1.860
8.3	25	0.219	0.612
16.6	50	0.218	0.547
33.3	100	0.219	0.552

TABLE VIII

*Solubilities of Phosphotungstates at 22° in Presence of Different Concentrations of HCl*  
Excess PTA in supernatant solution, 50 gm. per liter.

HCl concentration	Solubilities in mm of amino acid per liter							
	Diamino acids					Monoamino acids		
	Arginine	Histidine	Lysine	Hydroxy-lysine	Cystine	Glycine	Proline	Tryptophane
N								
0	0.066	0.299			0.450			
0.25	0.069	0.390	0.055	0.6*	0.507	32.8	21.1	4.43
0.5	0.150	0.470	0.081		0.596	35.4	23.3	
1.0	0.217	0.547	0.084		0.566	37.0	25.3	
2.0	0.221	0.347	0.040		0.364	31.8	20.8	
3.0	0.100	0.233				27.1	16.4	

\* Approximate, from a previous paper (24); subject to possible revision from data with more highly purified material.

### *Effects of Temperature*

The temperature effects are shown in the curves of Fig. 1.<sup>2</sup> All the solubilities were measured by the "resolution" technique. Most striking is the

<sup>2</sup> The solubility values in Fig. 1 are given in terms of nitrogen rather than mm of amino acid for two reasons: (1) The solubility differences between mono- and diamino phosphotungstates are not so great in terms of nitrogen as in moles; hence it is easier



extraordinarily high temperature coefficient of cystine, compared with all the other amino acids. The diamino acids, however, all show below room temperature definitely higher temperature coefficients than the proline and glycine.

It would appear therefore that, so far as one can judge from the solubilities of the separate amino acids, the most complete separations of the diamino from the monoamino group should be obtainable by precipitation of the diamino acids in large volumes at low temperatures. For example, at 38° the molar solubility ratio, proline-lysine, is 350 and at 22°, 333, while at 0° the ratio is 740 (in terms of nitrogen the ratios are half as great).<sup>3</sup>

With arginine, it was impossible to get constant solubilities at 0°. The variation was due to the formation of a gelatinous rather than a crystalline precipitate in most of the saturations at 0°. When this occurred, the solubility increased. In one experiment in which the gelatinization did not occur, the solubility at 0° was only 0.026 mm per liter, while in other experiments, in which gelatinization did occur, the solubility was found in the neighborhood of 0.10 mm, 4 times as great. The 0° point for arginine given in Fig. 1 represents the solubility obtained when gelatinization did not occur. There seems to be less tendency to gelatinize when the arginine is precipitated with other diamino acids.

#### *Effect of Coprecipitation with Lysine and Arginine in Decreasing the Solubility of Histidine Phosphotungstate*

A study of coprecipitation of histidine with lysine and arginine showed that when the molar ratio of lysine or of arginine, or of their sum, to histidine exceeds unity the precipitation of the histidine becomes increasingly more complete as the ratio increases.<sup>4</sup> Apparently mixed phosphotungstates are formed. A similar behavior of lysine in carrying down the 10-

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to put the values for both groups on a single logarithmic chart in nitrogen terms. (2) The solubilities in nitrogen terms can be applied directly as corrections in nitrogen distribution analyses of protein hydrolysates (4-6).

<sup>3</sup> Tryptophane forms a more insoluble phosphotungstate than either proline or glycine (Fig. 1), but it appears that tryptophane can be neglected in estimating conditions for separating the di- and monoamino acids, because it has not been observed to occur in acid protein hydrolysates in sufficient amounts to be precipitated with the diamino acids. Thus Van Slyke (4) in analyses of seven proteins of widely varying nature found in no case any glyoxylic acid test for tryptophane in the solution obtained by redissolving the diamino acid phosphotungstates.

<sup>4</sup> Our attention was first drawn to this phenomenon by finding in gelatin analyses (unpublished) that the precipitation of the diamino acid phosphotungstates left only one-third as much histidine in the filtrate as would be predicted by solubility determinations on pure histidine phosphotungstate.

fold more soluble hydroxylysine phosphotungstate has already been noted (24).

Fortunately for the utility of phosphotungstic acid in separating the diamino and monoamino fractions, the tendency of the diamino phos-

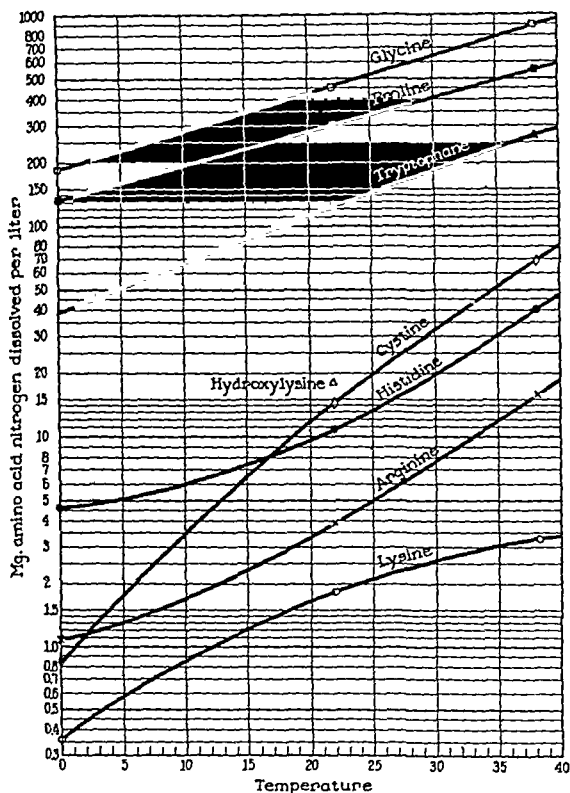


FIG. 1. Effect of temperature on the solubilities of amino acid phosphotungstates in the presence of 0.25 N HCl. The curve for histidine is not valid when the molar sum of arginine and lysine precipitated exceeds the histidine. When histidine is precipitated together with lysine or arginine or both, the solubility correction for histidine is to be estimated from Fig. 2.

photungstates to carry down the monoamino group is slight compared with the tendency of arginine and lysine to carry down histidine.

The experiments recorded in Tables IX to XII were performed by the "precipitation" technique. The lysine and histidine used were dichlorides,

the arginine a monochloride; all had been found to be pure by solubility tests as well as analysis.

The results summarized in Fig. 2 show that arginine and lysine have nearly equal effects in carrying down histidine with their phosphotung-

TABLE IX

*Solubilities of Phosphotungstates of Lysine and Histidine Precipitated Together*

Temperature, 22°; HCl in supernatant, 0.25 N; excess PTA in supernatant, 16.6 mM, 50 gm. per liter; solubilities determined by 48 hour precipitation and analysis of the supernatant solution.

Amino acids present in system, per liter		Left in 1 liter supernatant solution after pptn. (solubilities)		Pptd. per liter solution		Molar fraction of amino acids as histidine in ppt.
Histidine	Lysine	Histidine (photometric)	Lysine $\frac{(\text{NH}_2)}{2} - c$	Histidine, $a - c$	Lysine, $b - d$	$\frac{\text{Histidine} + \text{lysine}}{e + f}$
(a)	(b)	(c)	(d)	(e)	(f)	(g)
mM	mM	mM	mM	mM	mM	
3.89	0	0.390	0	3.50	0	1.00
3.89	1.01	0.393	0.039	3.50	0.97	0.78
3.89	1.01	0.392	0.038	3.50	0.97	
3.89	4.03	0.376	0.042	3.51	3.99	0.47
2.19	4.75	0.245	(0.04)*	1.94	(4.71)*	0.29
0.97	4.03	0.092	0.049	0.88	3.98	0.18
0.97	4.03	0.098	0.047	0.87	3.98	
0.55	4.75	0.033	(0.05)*	0.52	4.70	0.099
0.27	4.75	0.014	(0.05)*	0.26	(4.70)*	0.052
0	4.03	0	0.051	0	3.98	0

\* Interpolated.

states. On the other hand, histidine has little effect on the solubilities of the more insoluble phosphotungstates of lysine and arginine.

#### CONDITIONS FOR PRECIPITATION OF THE DIAMINO ACID PHOSPHOTUNGSTATES IN PROTEIN HYDROLYSATES

From data in this paper it appears that 0.25 N HCl offers a better medium than the previously employed 1 N HCl for separating the phosphotungstates

of the mono- from the diamino acids. It is also evident from Fig. 1 that in estimating the minimum volumes of solution that will keep glycine and proline phosphotungstates in solution during the precipitation<sup>3</sup> it is necessary to take temperature into account. We have indicated in Fig. 3 the volumes of 0.25 N HCl containing 50 gm. of free PTA per liter which are required to hold in solution the 0.26 gm. of glycine yielded by hydrolysis of 1 gm. of gelatin (29). Fig. 3 also indicates the amounts of PTA that must be added to provide the maximum (estimated at 2.5 gm. of PTA per gm. of protein) required to combine with the diamino acids and leave an

TABLE X

*Solubility of Histidine Phosphotungstate Precipitated with Lysine in a Molar Ratio of 4:1, with Varying Amounts of Substrate*

Temperature, 22°; the supernatant solution contained HCl in 0.25 N and PTA in 16.6 mM concentration; the solubilities were determined by 48 hour precipitation. The results show that the amount of mixed substrate present does not affect the solubility if the composition of the substrate is constant.

Amino acids present in system, per liter		Histidine in supernatant solution, per liter (photometric)	Molar fraction of amino acids as histidine in ppt.* $\frac{\text{Histidine}}{\text{Histidine} + \text{lysine}}$
Histidine	Lysine		
mM	mM	mM	mM
9.12	36.6	0.075	0.802
9.12	36.6	0.086	0.802
4.56	18.3	0.087	0.803
4.56	18.3	0.086	0.803
2.28	9.14	0.091	0.806
2.28	9.14	0.093	0.806
1.14	4.57	0.090	0.812
1.14	4.57	0.086	0.812

\* Calculated as in Table VIII, with the assumption that the lysine in the supernatant solution is 0.04 mM per liter.

excess of approximately 50 gm. of PTA per liter in the supernatant solution. Gelatin, because of its high glycine and proline contents, 27 and 18 per cent respectively (29), offers the most difficult mixture for the phosphotungstate separation that the writers have encountered, and it appears that conditions which suffice for the separation in gelatin will be adequate for protein analyses in general.

The conditions indicated in Fig. 3 have been tested on gelatin, fibrin, and edestin, and have been found to yield a precipitate of diamino acids free of monoamino acids, except small amounts of the latter adsorbed by the precipitate.

These cannot be removed by washing, but are removed by one recrystallization of the diamino phosphotungstates. For recrystallization the phosphotungstates can be redissolved by boiling water, or in the cold by adding just enough 1 N NaOH to a water suspension of the phosphotungstates to dissolve them ((8) pp. 267, 268). In either case enough HCl is then added to bring its concentration (in excess of any NaOH added) to

TABLE XI

*Solubilities of Phosphotungstates of Arginine and Histidine Precipitated Together*

Temperature, 22°; HCl in supernatant, 0.25 N; PTA in supernatant, 16.6 mM, 50 gm. per liter; solubilities determined by 48 hour precipitation and determination of histidine (photometric) and total nitrogen in the supernatant solutions.

Amino acids present in system, per liter		Solubilities, amino acids left in 1 liter supernatant		Pptd. per liter solution		Molar fraction of amino acids as histidine in ppt.
Histidine	Arginine	Histidine (photometric)	Arginine $\frac{\text{Total N} - 42c}{56}$	Histidine, $a - c$	Arginine, $b - d$	$\frac{\text{Histidine} + \text{Arginine}}{a + b}$
(a)	(b)	(c)	(d)	(e)	(f)	(g)
mM	mM	mM	mM	mM	mM	
+	0	0.39*		+	0	1.00
3.90	1.055	0.399	0.078	3.50	0.98	0.78
4.39	4.75	0.362	(0.08)†	4.03	4.67	0.46
3.90	4.22	0.390	0.070	3.51	4.15	0.46
2.19	4.75	0.212	(0.08)†	1.98	4.67	0.30
1.10	4.75	0.081	(0.08)†	1.02	4.67	0.179
0.98	4.22	0.103	0.090	0.88	4.13	0.175
0.55	4.75	0.043	(0.08)†	0.51	4.67	0.099
0.27	4.75	0.019	(0.08)†	0.25	4.67	0.051
0	+		0.075*	0	+	0

\* Results in the top and bottom rows for solubilities of histidine and arginine precipitated alone are from previous experiments.

† The solubilities of arginine in parentheses are interpolated. The total nitrogen in the supernatant was not determined.

0.25 N. Then 50 gm. of PTA per liter are added, in order to effect the recrystallization under the same solubility conditions as the first precipitation.

In applying solubility corrections for the diamino acid phosphotungstates in protein hydrolyses, the corrections for arginine and lysine may be obtained from Fig. 1. For histidine the correction must be taken from Fig. 2 after the proportions of lysine, arginine, and histidine in the precipitate have been determined.

TABLE XII

*Solubility of Histidine Phosphotungstate Precipitated in Varying Proportions with a Mixture of Arginine and Lysine*

Temperature 22°; the supernatant solution contained HCl in 0.25 N and PTA in 16.6 mM concentration; the solubilities were determined by 48 hour precipitation.

Amino acids present in system, per liter			Histidine in supernatant solution, per liter (photometric)	Molar ratio in ppt.* Histidine Histidine + arginine + lysine
Histidine	Lysine	Arginine		
mM	mM	mM	mM	
			(0.39)†	(1.00)†
4.38	2.37	2.37	0.390	0.46
2.19	2.37	2.37	0.244	0.29
1.10	2.37	2.37	0.124	0.18
0.55	2.37	2.37	0.083	0.09
0.27	2.37	2.37	0.046	0.046

\* Calculated as in Table VIII, with the assumption that, of the 2.37 mM each of lysine and arginine present, 2.33 mM of the lysine and 2.30 of the arginine were precipitated.

† Solubility of histidine phosphotungstate in the absence of other amino acids, from other experiments.

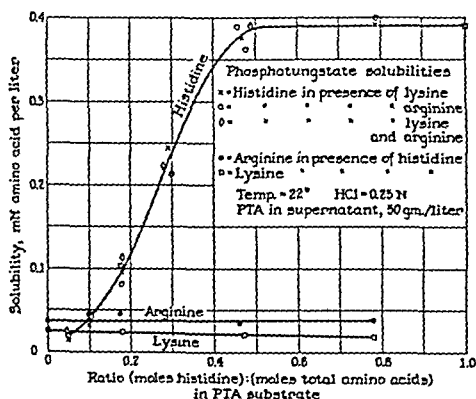


FIG. 2. Solubility of histidine phosphotungstate in the presence of varying proportions of lysine and arginine phosphotungstates. As the proportion of histidine in the diamino acid mixture falls below half, histidine precipitation becomes progressively more complete. The effect is not reciprocal: large proportions of histidine have little effect on the solubilities of arginine and lysine (two lower curves).

For the histidine determination we find that direct colorimetric determination by the method of Jorpes (22) applied to the solution of diamino phosphotungstates is more exact than the calculation by difference origi-

nally used by Van Slyke (4, 6). Calculation of lysine from the difference between amino nitrogen and carboxyl nitrogen, as described on p. 658 of Van Slyke, Dillon, MacFadyen, and Hamilton (23), is more exact than the calculation originally used (4); the lysine calculation from amino and carboxyl nitrogens also has the advantage that it is not affected by the presence of cystine in the diamino mixture, nor by adsorbed monoaminomonocarboxylic acids, such as glycine. These methods for histidine and lysine, as well as the original alkali-decomposition method for arginine (4) can be applied to the solution obtained by simply redissolving the di-

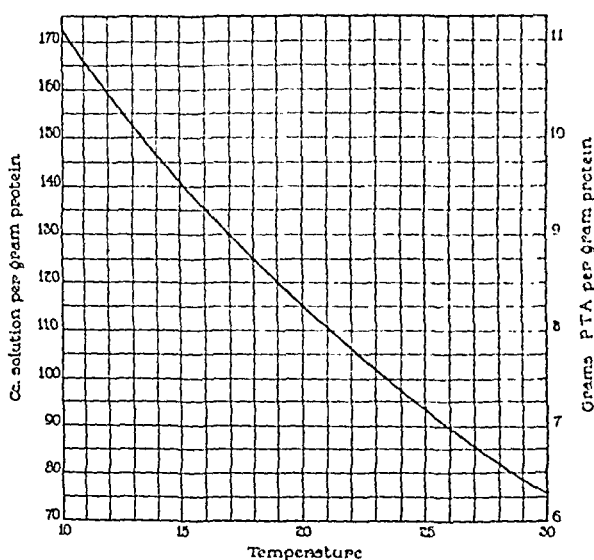


FIG. 3. Volumes of 0.25 *N* HCl and amounts of PTA, per gm. of protein, which appear to give optimal conditions for separation of the diamino from the monoamino acids in protein hydrolysates.

amino acid precipitate in enough alkali to give a neutral solution, without removing the PTA.

#### SUMMARY

The solubilities of the salts of 12-phosphotungstic acid formed by the diamino acids, arginine, histidine, lysine, and cystine, and of the monoamino acids, glycine, proline, and tryptophane, have been measured under varying conditions of temperature and mineral acid concentration, and what appear to approximate optimal conditions have been ascertained for the phosphotungstate separation of the diamino from the monoamino acids in protein hydrolysates.

Histidine has been found to form mixed phosphotungstates with arginine

and lysine, so that when the molar sum of lysine and arginine exceeds the histidine, precipitation of the histidine is more complete than indicated by the solubility of its isolated phosphotungstate. The solubility effect of the arginine and lysine on the histidine has been plotted as a function of the proportion of histidine in the mixture, so that solubility corrections can be made for histidine as well as the other two diamino acids. Histidine does not show a reciprocal effect on the solubilities of lysine and arginine phosphotungstates.

Under the conditions used, the amino acid phosphotungstates precipitated, except that of cystine, approximate in composition the trivalent formula of previous authors, 3 moles of a monoamino acid, or 1.5 moles of a diamino acid, precipitating with 1 mole of  $\text{H}_3\text{PO}_4 \cdot 12\text{WO}_3$ .

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# ANALYZER FOR QUICK ESTIMATION OF RESPIRATORY GASES

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The present method was developed in order to meet conditions under which the speed and convenience of a less accurate method are of greater importance than the high accuracy obtained in the time-consuming methods of ordinary gas analysis. An analysis with the method presented takes only from 0.5 to 5 minutes and gives results accurate to 0.5 to 1 per cent of the total volume analyzed.

*Principle*—The gas sample is drawn through a hypodermic needle into a syringe which is adjusted to a fixed capacity. The gas is injected through a rubber tube directly into a measuring burette which is filled with the desired absorbent. The percentage of gas left or absorbed is read directly on the burette.

*Apparatus*—The apparatus consists of two burettes, each connected through a rubber tube with a leveling bulb, and of two 5 cc. syringes in adjustable holders. The apparatus is conveniently mounted on a panel, as shown in Fig. 1. The burettes are of 5.5 mm. bore and are graduated in 100 parts from the closed end, so that the graduated capacity is 5 cc. At the open end of the burette is a bulb of about 25 cc. capacity. For determining very low percentages of nitrogen, it is an advantage to have the top of one burette constricted as in *B*. The burette is connected through a heavy rubber tube of around 9 mm. bore with the leveling bulb of 15 to 20 cc. capacity. The opening of the burette and leveling bulb into the rubber tube should be kept full size. The leveling bulbs are fastened securely to the panel by means of a metal strap which can be tightened with a wing-nut on the back side of the panel. The burette hangs freely down in the rubber tube, or it can be arrested in a fuse clip at the lower end. Between the leveling bulbs are fastened two pairs of fuse clips fitting the burette stem. The leveling bulbs are provided with grooved stoppers.

For gas samplers two 5 cc. syringes are used which are fixed rigidly in a frame (see Fig. 1, *C*), so that their maximum capacity can be adjusted and fixed by a set-screw and lock-nut to match the graduated capacity of the respective burette. It is essential that the set-screw should engage the center of the plunger head. If that is not the case, care must be taken that the syringe plunger is always turned in the same direction when hitting the screw. The frames should be made of plastic or other heat-insulating

material. It is advisable to put a piece of insulating tape between the syringe and the frame before tightening the screw-hook which holds the syringe fixed. The syringes are provided with short, fine hypodermic needles (page 24).

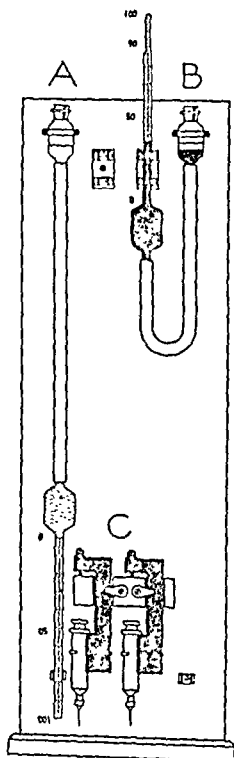


FIG. 1. Apparatus fastened to its panel. *A* is filled with potassium hydroxide and *B* with hydrosulfite. *B* shows the position of the burette when the gas is squirted in through the rubber tube below the burette bulb and also the position at which the final gas volume is read; that is, with the liquid menisci level. *A* shows the position of the other burette between the analyses when the gas of the burette has escaped up through the rubber tube and leveling bulb. The graduation and numbering of the burette tubes are indicated. *C*, syringe samplers fixed to a bakelite frame provided with set-screws and lock-nuts for the accurate adjustment of the delivery capacity of the syringes. The samplers may be hung on the panel when not in use, as indicated.

*Reagents*—*A* is filled with 10 per cent potassium hydroxide solution for the carbon dioxide absorption. *B* is filled with 10 per cent potassium hydroxide solution containing 15 per cent dry hydrosulfite-anthraquinone- $\beta$ -sulfonate mixture (10:1). The solution should be made up in a stoppered

bottle which it completely fills. A layer of mineral oil prevents the solution from being oxidized in the leveling bulb. A beaker with 2 per cent sulfuric acid containing an indicator completes the reagents.<sup>1</sup>

*Calibration*—The vaseline-greased syringe is filled with atmospheric air; care should be taken not to touch and heat the chamber walls of the syringe. The burette (*A*) is held upright, as in *B*, and the rubber tube underneath the burette is pierced obliquely with the syringe needle and the gas squirted into the alkali. The gas volume collects at the top of the burette and is read with the burette meniscus and the leveling bulb meniscus level. The burette is hung down, whereby the gas escapes up through the rubber tube and out through the leveling bulb. The syringe is rinsed with a little acid and the set-screw is adjusted so that after some few repetitions the volume expelled from the syringe comes exactly to the 0 mark of the burette, after sufficient time for drainage is allowed. The same procedure is used for the burette with hydrosulfite and the other syringe. In this, however, the set-screw is adjusted so as to give a gas reading in the burette of exactly 79, which is the nitrogen percentage of atmospheric air.

### *Procedure*

For securing the gas sample it will usually be possible to arrange it so that the gas can be taken by puncturing a rubber tube with the syringe needle. The syringe is filled with the gas by repeated washing, during which one is careful not to warm the syringe chamber by claspings it in the hand. The needle is taken out and immediately stuck into the analyzer, which is gas-free and held in an upright position (*B*). The gas is slowly squirted in and the volume read after the liquid has drained from the walls and the menisci have been leveled. The burette is freed of gas for the next analysis by hanging it down as in (*A*). The syringe is washed with a little acid before the next analysis. If nitrogen is determined in samples with high oxygen content, 0.5 cc. of mercury may be put into *B*. The gas is introduced slowly, and the bubbles together with the mercury are shaken violently in the bulb of the burette, while it is kept horizontal and in a high position. The mercury helps to break up the crust which is formed around the bubbles and thus speeds up the absorption. Tank oxygen, for instance, requires shaking in this way, in order to be absorbed quickly. Shaking is continued until the reading remains constant.

Oxygen is determined on two independent samples, in the one of which the carbon dioxide is determined and in the other the carbon dioxide plus

<sup>1</sup> The water vapor tensions of these reagents differ less than 2 mm., according to determinations with the Van Slyke manometric apparatus. This gives less than 0.25 per cent error in the volumetric readings, and accordingly is negligible.

the oxygen. The difference between the readings gives the percentage of oxygen.

If the samples cannot be analyzed at once, they can be stored for a short time in the syringes by putting the syringe tip in a little water, as a seal.

For special purposes, it may be sufficient to have only one burette, as in determination of the amount of air leaking into an oxygen chamber during oxygen administration; it may then also be an advantage to calibrate the burette in reverse of what is shown in the picture, so that the nitrogen percentage is directly read. In most cases of ordinary laboratory work, it is preferable to have both burettes like *A*, without a constricted tip. Less than 1 per cent of residual gas can still be determined by injecting several syringes. By leaving a small known amount of gas in the burette from the preceding analysis, the poor definition of reading small amounts of nitrogen, in analyses of  $O_2$ -enriched air, near the tip of the burette can be avoided.

I wish to express my gratitude to Dr. Laurence Irving for his helpful support.

The apparatus was made by Mr. J. D. Graham.

# THE ACID-BASE-BINDING CAPACITY OF COLLAGEN

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(Received for publication, September 1, 1942)

Atkin (1) in 1933 discussed the composition of gelatin and collagen and came to the conclusion that its molecular weight should be 34,500. On the basis of data taken from that paper and other later sources, it is possible to make further calculations (2, 3) as shown in Table I. These data would appear to indicate that if the amounts of arginine, lysine, and histidine are taken into account, then such a sum would agree rather well with the maximum amount of acid bound as determined by a number of workers. However, if the sum of the aspartic acid, glutamic acid, and tyrosine is considered in relation to the maximum base binding as found by these same workers, agreement is extremely poor. The work of Hitchcock (4), dealing with the acid-base-binding capacity of gelatin as measured by potentiometric methods would seem to indicate a maximum acid binding of approximately 0.96 milliequivalent per gm. of protein, an isoelectric point at pH 4.7 to 4.8, a broad plateau region between pH 6.5 and 8.5, and an approximate maximum base fixation of 0.95 milliequivalent per gm. of protein. Curves taken from the work of Jordan Lloyd and Bidder (5) show only an indication of an acid-binding maximum better than 0.9 milliequivalent per gm. of protein, a broad isoelectric zone, a broad plateau in the pH range 8 to 11, and a base-binding maximum of better than 0.8 milliequivalent at pH 13. The curve representing data employed by Highberger (6) using highly purified collagen is quite different from similar curves obtained by any of the other investigators in this field. Highberger has pointed out that the validity of applying the potentiometric methods used for homogeneous solution is rather open to question. His curve for the purified collagen showed no indication of a maximum acid fixation but an actual decreased acid binding at pH values less than 2, an isoelectric zone in the pH range 4.9 to 6.0, a very broad plateau in the range pH 7 to pH 10.1, and no real values for maximum base binding in the more alkaline range. Curves obtained by Theis and Stubbings<sup>1</sup> for collagen using a combination potentiometric and titration method yielded low and erratic values for maximum acid fixation and base fixation. Highberger (6) ascribed such discrepancies as due to variation in the ratio of acid and water absorbed by the protein.

<sup>1</sup> Theis, E. R., and Stubbings, R. L., unpublished data.

The methods used by most investigators in determining the titration curve for collagen have been modifications of the classical potentiometric one, in which many thermodynamic assumptions must of necessity be made. While the potentiometric method may be extremely useful in determining the acid-base fixation of soluble proteins, the present writers believe that it is not applicable to fibrous proteins, since such systems are heterogeneous systems. Sheppard, Hauck, and Dittmar (7) used a dye adsorption technique and found for gelatin two different acid fixation values. For alkali-processed gelatin approximately 0.9 milliequivalent of HCl was fixed, while for acid-processed gelatin 1.5 milliequivalents of acid were bound.

McLaughlin and Adams (8) in 1940 introduced a certain technique in protein analysis. Their method was to treat the protein in question with

TABLE I  
*Amino Acid Content of Collagen*

Amino acid	Amino acid in collagen	
	<i>per cent</i>	<i>mm per gm.</i>
1. Aspartic acid .....	3.4	0.257
2. Glutamic " .....	5.8	0.394
3. Tyrosine.....	1.0	0.055
4. Amide N.....	0.5	0.358
(1) + (2) - (4) .....		0.293
(1) + (2) + (3) - (4).....		0.348
5. Arginine.....	8.2	0.471
6. Lysine .....	5.0	0.343
7. Histidine.....	0.9	0.060
(5) + (6) + (7).....		0.874
(5) + (6) .....		0.814

acid and, after equilibrium had been established, press the protein twice at some 5000 pounds per sq. inch pressure. This treatment, these workers suggested, removed all free water and free electrolyte. After being pressed, the protein material was air-dried and ground to a fine powder previous to analysis. Jordan Lloyd and Moran (9) have also postulated that a pressure of some 8000 pounds per sq. inch will remove all free water. Theis and Jacoby (10) in 1941 investigated this pressing technique rather thoroughly and came to the conclusion that acid- or base-treated collagen could be pressed free of all unbound electrolyte. In a published work in 1941, Theis and Jacoby gave a preliminary method for using this pressing technique in obtaining the acid- or base-binding capacity of silk fibroin, keratin, and collagen. In that work, however, an equilibrium period of 72 hours was used, which in later work was found to be too

severe and too long in the low and high pH ranges. As a consequence, certain modifications of technique and methods were made which now, the writers believe, give a true picture of the acid-base-binding capacity of collagen over the pH range 0.5 to 13.5.

*Preparation of Collagen Material*—Goatskins were carefully unhaired by being immersed in a solution of calcium hydroxide containing excess solid calcium hydroxide for 48 hours. The loosened hair was then carefully scraped off and the skins thoroughly washed with running water. After being washed, the skins were placed in a weak solution of acetic acid (pH 4), thoroughly delimed, and again washed with running water. After such treatment, the skins were dehydrated by means of several changes of acetone followed by several washings with alcohol. The dehydrated skins were then air-dried for several days and cut into pieces 0.5 by 1.5 inches. The thoroughly dried and mixed pieces of skin were practically ash-free and were the source of the collagen material used for the experiments outlined in this paper.

#### EXPERIMENTAL

1 gm. pieces of the collagen material were placed in 200 ml. bottles and 100 ml. of various concentrations of hydrochloric acid or potassium hydroxide were then added. The concentrations were such that at equilibrium the pH values would vary between 0.5 and 13. The bottles and contents were then placed in a thermostat maintained at 20° for a prescribed period. After equilibrium had been attained, the equilibrium pH was measured by means of a Beckman glass electrode assembly. The pieces of treated collagen were then pressed several times at 10,000 pounds per sq. inch in a Carver press. After being pressed, the collagen pieces were air-dried and ground in a small Wiley mill and were then ready for analysis. The hydrogen or hydroxyl ion was determined by a method to be described. The total nitrogen was determined by a semimicromethod in which 0.1 gm. of the ground material was used.

The method for determining  $H^+$  or  $OH^-$  ion was as follows: 0.5 gm. of the ground material was carefully weighed into a 250 ml. Erlenmeyer flask. To this were added 50 ml. of distilled water and 10 ml. of 0.1 N HCl. The added HCl aids materially in hydrating the acid-treated collagen and in the case of the alkali-treated collagen neutralizes the fixed or bound base and thus converts the protein-base compound into an acid-protein compound. This addition of the HCl is essential for correct results. After a 2 hour treatment as described, 10 ml. of a potassium iodine-potassium iodate solution (containing 200 gm. of KI and 50 gm. of  $KIO_3$  per liter) are added together with 20 ml. of 0.1 N sodium thiosulfate and the reagents allowed to react for approximately 2 hours. The excess



0.1 N  $\text{Na}_2\text{S}_2\text{O}_3$  is then titrated back with 0.1 N HCl or 0.1 N iodine solution. A blank determination containing all the reagents is run with each set of experiments. The volume of 0.1 N  $\text{Na}_2\text{S}_2\text{O}_3$  consumed is then calculated to the  $\text{H}^+$  ion or  $\text{OH}^-$  ion fixed per gm. of protein.

This method is extremely accurate and is comparatively simple. No assumptions need be made and the protein used for the experiment need not be ash-, acid-, or alkali-free, since only the acid or alkali fixed is measured by the method. This method has many advantages over the potentiometric one, since it can be used for the heterogeneous system (fibrous protein-acid) and the amount of acid fixed at any hydrogen ion concentration is not a thermodynamic calculation but is an actual quantitative determination upon the treated protein.

### Results

Fig. 1 shows in graphical form two titration curves over a wide pH range. These values were obtained by allowing the samples of collagen to attain equilibrium with their respective solutions over a period of 72 hours. Curve A represents aqueous acid or base solution while Curve B represents acid or base solution maintained 0.1 N with respect to potassium chloride. Both curves indicate a maximum acid fixation of approximately 0.94 milliequivalent per gm. of protein at pH values less than 1.0 and a maximum alkali fixation of approximately 0.46 milliequivalent per gm. of protein at pH values greater than 12.8. The curve for the aqueous solutions (Curve A) shows a sharp slope in the pH range 2.0 to 4.2, a broad isoelectric zone in the pH range 4.6 to 7.5, a rather broad plateau in the alkali fixation range pH 8.0 to 11.0, and a sharp increase in alkali fixation at pH values of 11.0 to 12.0. Curve B representing salt solutions of acid or base, while showing the same maximum acid or base fixation, has an entirely different trend in other sections of the curve. This curve shows a much decreased slope in the pH range 2.0 to 6.0, a definite isoelectric point at pH 7.6, and a much increased alkali binding in the pH range 8 to 10, with a rather flat region between pH 10 and 12.

The analysis of the collagen for arginine, lysine, histidine, aspartic acid, glutamic acid, and tyrosine indicates that there should be a maximum acid fixation of approximately 0.874 milliequivalent per gm. of protein and a maximum base fixation of 0.348 milliequivalent per gm. of protein. The curves pictured in Fig. 1 show approximately an 11 per cent greater value for acid fixation and some 30 per cent greater values for base fixation. The values for maximum acid fixation, however, are approximately those obtained by Hitchcock for gelatin. However, with regard to base fixation, the values shown in Fig. 1 are decidedly more in line with analytical data than those given by Hitchcock (4), by Jordan Lloyd and Bidder (5), and by Highberger (6).

The displacement of the titration curves with potassium chloride solution rather than a mere aqueous solution is undoubtedly due to the fact that the salt causes a more uniform diffusion of the acid or base into the collagen and can be readily explained by the Donnan membrane equilibrium theory. By using potassium chloride solutions of acid or base, an absolute isoelectric point can be attained as shown by Curve B of Fig. 1. Beek and Sookne (11), Highberger (12), and Theis and Jacoby (13) have shown by electrophoretic studies that the true isoelectric point of collagen is pH 7.6 to 7.8. However, titration curves for collagen have always indicated an isoelectric point approximating pH 5 and it has been

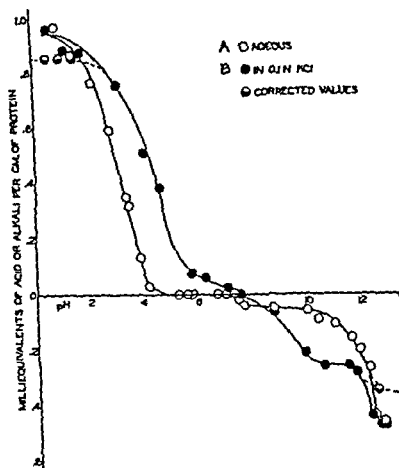


FIG. 1. The titration curve of collagen, showing the uncorrected and corrected values in the high acid and high alkaline region. The dotted portions of the curve show the most probable values of maximum acid- or base-binding capacity of the collagen.

difficult to correlate values obtained by this means with those obtained by electrophoretic means. Curve B shows rather clearly that the isoelectric point given by our present method of obtaining such titration curves agrees well with values obtained by electrophoresis studies.

The writers feel that the method outlined above for obtaining acid or base fixation of fibrous proteins is a more dependable one than the potentiometric method usually employed, since no assumptions are made other than that high pressure removes all free electrolyte. However, at pH values less than 2 and greater than 12, the writers believe that the data indicate greater amounts of acid or base fixed than is actually the case for unaltered protein, and that this increase is due to hydrolysis and simplifica-

tion of the protein in those pH ranges. Since the data for Fig. 1 were for a 72 hour equilibrium period, the writers studied the acid and base fixation at pH 0.85 and 12.5 over a period of 100 hours. Curves A and B of Fig. 2 show data obtained in this way. Fig. 2 shows clearly that hydrolysis occurs after some 20 hours, after which the fixation of acid or base increases sharply. Such results indicate that in the more acid and in the more alkaline regions a shorter period of reaction must be used. The dotted portions of the curve given in Fig. 1 show the most probable values of maximum acid- or maximum base-binding capacity of the collagen. These maximum values show 0.85 milliequivalent of acid bound per gm. of protein and 0.35 milliequivalent of base bound per gm. of protein.

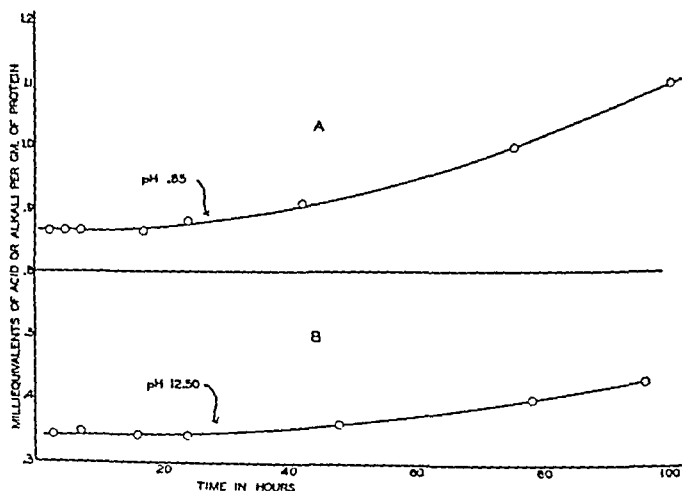


FIG. 2. The increased acid or base binding by collagen due to hydrolysis

Analytical data given in Table I indicate that 0.874 milliequivalent of acid and 0.345 milliequivalent per gm. of protein should be fixed.

#### SUMMARY

A method for determining the acid-base-binding capacity of fibrous proteins is given in which free water and uncombined electrolyte are removed by means of pressure. The acid or base actually fixed with the protein is then determined by a modification of an iodometric method. The results obtained are well in line with other analytical data.

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# A METHOD FOR THE QUANTITATIVE DETERMINATION OF HEMOGLOBIN AND RELATED HEME PIGMENTS IN FECES, URINE, AND BLOOD PLASMA\*

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The purpose of the present study was to make available a simple quantitative procedure for total heme pigments in feces, urine, and blood plasma or serum. It was planned to convert these substances to pyridine ferrohemochromogen, which is known to have stronger absorption than other easily prepared heme compounds, and to measure this absorption under standard conditions with the Evelyn photoelectric colorimeter.

Stokes (1) discovered the hemochromogen spectrum and Hoppe-Seyler (2) gave the substance its name. Anson and Mirsky (3) defined a hemochromogen as a substance containing reduced heme and a nitrogen compound. Pyridine ferrohemochromogen has absorption bands at 5575 Å. (maximum) and 5270 Å. The first band is stronger than that of any other heme compound, having an extinction coefficient about one-third greater than that of oxyhemoglobin (4). An equilibrium is reached; so that it is necessary to have an excess of pyridine in order to complete the reaction (3).

Hill (5) used a micro spectroscope mounted on a colorimeter to determine pyridine hemochromogen concentrations. He compared unknown solutions with a standard in order to get information about the equilibrium reactions of hemochromogen. Anson and Mirsky (6) also used the micro spectroscope and colorimeter for determining the amount of cytochrome in yeast. Lemberg and coworkers (7) used the same apparatus to follow the course of the coupled oxidation-reduction reaction of ascorbic acid and hemochromogens. Roetts (8) described a method of determining the content of hemoglobin and related substances in feces, urine, serum, and milk by forming pyridine hemochromogen and comparing the absorption with that of a standard solution.

## Method

The following method for determining the content of hemoglobin and related heme compounds in feces, urine, and serum or plasma has been devised. The method entails the use of the Evelyn photoelectric colorimeter. A calibra-

\* Aided by grants from the C. P. DeLaittre Research Fund and from the Medical Research Fund of the Graduate School, University of Minnesota.

tion curve was prepared by making eighteen dilutions of blood ranging from 0.06 to 3.0 mg. of hemoglobin in eighteen Evelyn colorimeter tubes. To each tube were added 2 cc. of pyridine, 2 cc. of freshly prepared clear aqueous 2 per cent  $\text{Na}_2\text{S}_2\text{O}_4$ , and enough 10 per cent  $\text{NH}_4\text{OH}$  to make up the volume to 10 cc. After 5 minutes the galvanometer reading was obtained; a 550  $\text{m}\mu$  filter and a center setting from a blank tube were used. A straight line graph relating the concentration of hemoglobin to the

TABLE I

*Data for Calibration Curve for Pyridine Ferrohemochromogen with Photoelectric Colorimeter*

Hemoglobin	Galvanometer reading	Hemoglobin	Galvanometer reading
mg.		mg.	
0.06	97.25	0.6	77.25
0.12	93.50	0.9	68.0
0.18	91.25	1.2	58.0
0.24	89.50	1.5	53.0
0.30	87.25	1.8	46.0
0.36	85.50	2.1	40.75
0.42	82.75	2.4	36.75
0.48	81.50	2.7	32.0
0.54	78.50	3.0	28.0

logarithm of the galvanometer reading was obtained. The following formula is then used. (See Table I.)

$$\begin{aligned}
 M &= VL/K_1 \text{ mg. hemoglobin in sample contained in colorimeter tube} \\
 L &= (2 - \log G) = L \text{ value} \\
 G &= \text{galvanometer reading} \\
 V &= \text{cc. in colorimeter tube} \\
 K_1 &= 1.843
 \end{aligned}$$

*Plasma Hemoglobin Content*—Obviously plasma must be obtained without artificially induced hemolysis. Ham (9) recommends the use of a clean syringe rinsed with physiological saline, withdrawal of blood without venous stasis, and gentle emptying of the syringe into a graduated centrifuge tube containing 3 per cent sodium citrate solution (about 0.1 volume). The blood is then centrifuged immediately and the plasma withdrawn. (Correction for the volume of citrate must be made by multiplying the result of the determination by the volume of supernatant fluid divided by the volume of supernatant fluid minus the volume of citrate.) 1 cc. of plasma is placed in an Evelyn colorimeter tube. It is first diluted with 10 per cent  $\text{NH}_4\text{OH}$  to 7.5 cc. and finally to 10 cc. with 0.5 cc. of pyridine and 2.0 cc. of freshly prepared 2 per cent  $\text{Na}_2\text{S}_2\text{O}_4$ . A

duplicate tube is set up in a similar manner with 1.0 cc. less  $\text{NH}_4\text{OH}$ , thus allowing for 1.0 cc. of 3 per cent  $\text{H}_2\text{O}_2$ . The center setting is determined on the duplicate after 5 minutes are allowed for decolorization. The galvanometer reading of the unknown solution is then determined. The hemoglobin content can be calculated readily from the calibration curve (Table I).

*Hemoglobin Content of Feces*—(1) The total amount of feces is weighed and thoroughly mixed, and a small portion of it (1 to 10 gm., depending upon the apparent content of hemoglobin or hematin) is weighed in an evaporating dish, (2) extracted with ethyl ether or acetone to remove fats, (3) acidified with 2 to 4 cc. of 5 per cent  $\text{HCl}$  and 1 cc. of glacial acetic acid, and (4) extracted four times by thorough grinding with 25 cc. of a mixture of 1 part of 95 per cent ethyl alcohol and 4 parts of peroxide-free ethyl ether. (5) The extract is decanted, filtered, and then washed with water in a separatory funnel. (6) The ether extract is then alkalinized with 10 per cent  $\text{NH}_4\text{OH}$ . The heme pigments separate out quantitatively into the aqueous phase during two or three extractions. (7) The extract is collected in an appropriate volumetric flask (25 to 100 cc.) and diluted to the mark with water. (8) 1 to 2 cc. of the final extract is then placed in a colorimeter tube and 2 cc. of pyridine and 2 cc. of freshly prepared 2 per cent  $\text{Na}_2\text{S}_2\text{O}_4$  are then added, after which the volume is made up to 10 cc. with 10 per cent  $\text{NH}_4\text{OH}$ . (A duplicate tube for the center setting reading is prepared by decolorization with  $\text{H}_2\text{O}_2$ , as described above.) The galvanometer reading and the hemoglobin content of the sample are determined and the calculation is made as follows:

$$\frac{M}{1000} \times \frac{\text{volume extract}}{\text{volume aliquot}} \times \frac{\text{amount (volume or weight) of specimen}}{\text{amount (volume or weight) of sample}} = \text{gm. Hb}$$

*Urine Hemoglobin Content*—The volume of the urine specimen is determined and, depending on the apparent quantity of hemoglobin in the specimen, 10 to 100 cc. of urine are placed in a separatory funnel and acidified with 5 cc. of 5 per cent  $\text{HCl}$ . Four extractions with 25 to 30 cc. of the alcohol-ether mixture described above are carried out. (Emulsions can usually be broken by adding small amounts of 95 per cent ethyl alcohol.) The alcohol-ether extract is washed once with water, after which one proceeds as in the method for feces above.

#### EXPERIMENTAL

*Plasma*—The plasma of twenty normal individuals contained less than 7.5 mg. of hemoglobin per 100 cc. Recovery experiments have been carried out repeatedly by adding known amounts of blood to plasma.



With amounts of hemoglobin from 18.6 to 266 mg. per cent, the error in recovery has ranged from +9 to -10 per cent. For values below 10 mg. per cent the error has been as high as +25 per cent. See Table II for the data.

TABLE II  
*Recovery of Hemoglobin Added to Plasma, by Means of Quantitative Method*

Plasma	Hemoglobin added*	Amount determined	Error
cc.	mg.	mg.	per cent
1.0	0.064	0.08	+25
1.0	0.118	0.126	+7
1.0	0.186	0.19	+2
1.0	0.22	0.20	-9
1.0	0.234	0.235	+0.5
1.0	0.378	0.348	-9
0.5	0.33	0.33	0
0.5	0.50	0.51	+2
0.5	0.66	0.69	+5
1.0	1.068	1.1	+3
1.0	1.17	1.2	+3
1.0	1.47	1.5	+2
1.0	1.572	1.7	+9
1.0	2.67	2.66	-0.3

\* This value includes from 0.01 to 0.03 mg. in plasma as determined by the method on the plasma sample before the hemoglobin was added.

TABLE III  
*Plasma Hemoglobin Content in Patients with Transfusion Reactions*

Patient	Time after reaction	Symptoms and signs	Hemoglobin concentration
			mg. per cent
L. P.	1 hr.	Chill, 40°	1
A. K.	2 hrs.	" 40°, pulse 160	5
C. S.	30 min.	" 38.2°	6.2
M. M.	30 "	No chill, 37.8°	12
H. S.	1 hr.	Chill, 39.4°	2.2
M. K.	3 hrs.	" 39.8°	125
	5 "		75
A. Kr.	2 "	Fever, red urine (only urobilin on test)	4

Practical application of this method has been afforded chiefly in the determination of the presence or absence of hemolysis following blood transfusions. Seven patients with moderately severe chills and fever following transfusions were studied (see the data in Table III). Six

patients exhibited a plasma hemoglobin content of less than 12 mg. per cent. One patient, M. K., developed a chill and fever following a transfusion of 500 cc. of citrated blood. She apparently had no more severe reaction than the other patients, but her plasma hemoglobin content 3 hours after the reaction was 125 mg. per cent, and 5 hours after was 75 mg. per cent. No hemoglobinuria occurred, but jaundice developed in 12 hours as further evidence of a hemolytic process. It is often very difficult to assay the importance of hemolysis in transfusion reactions and one must ordinarily rely on the appearance of hemoglobinuria or jaundice or both. It would be helpful, therefore, to know shortly after the reaction whether or not hemoglobinemia is present; such a determination may come to be of considerable importance in prognosis and treatment of severe reactions. Simple inspection will serve to detect hemolysis when the concentration of hemoglobin is greater than 25 mg. per cent; so that one can recognize significant amounts of hemolysis by mere inspection of carefully drawn and separated plasma or serum. It is quite likely, however, that much more exact information especially as to prognosis can be gained with the quantitative method.

A sterile hemoglobin solution was injected on two occasions into a patient with aplastic anemia. It was found that hemoglobinuria persisted after the plasma level of hemoglobin had dropped below 50 mg. per cent, but no hemoglobin was excreted after the level dropped below 30 mg. per cent. The so called renal threshold for hemoglobin in normal persons has been found to average about 70 mg. per cent, although various authors (10, 11) have found a range of from 37 to over 200 mg. per cent (see the data in Table IV).

*Feces*—Putrefaction of hemoglobin in the intestinal tract causes varying amounts of decomposition. Deuterohematin is one of the products. The hemochromogen of deuterohematin has absorption bands at 5450 Å. and 5170 Å. By preparing crystalline deuterohemin (12) and forming a calibration curve, it was found that a maximum error of approximately 20 per cent would result if all hemoglobin were converted to this pigment (Table V). Snapper and van Creveld state that in occasional samples of feces studied by them most of the hemoglobin had been converted to deuterohematin. Although we have usually encountered small amounts of deuterohematin in feces containing blood, it has never constituted more than a small fraction of the total heme pigment present. This statement is based simply upon spectroscopic observations. Proto- and deuteroporphyrins may also be formed to a minor extent (12-15). The portion of hemoglobin which has been converted to porphyrin is not determined by this method.

Recovery experiments have resulted in 75 to 95 per cent recovery of

hemoglobin added to stool specimens (Table VI). The failure to recover all of the added hemoglobin has not been explained. At first it was thought that the fraction not recovered had been converted to proto- or deuteroporphyrin. By means of a Klett fluorophotometer, the total porphyrin content of a sample of feces was determined on each of two equal amounts, to one of which 60 mg. of hemoglobin had been added. Although recovery

TABLE IV

*Disappearance of Hemoglobin in Case of Aplastic Anemia, after Intravenous Injection*

Hb injected in physiological saline solution	Time after injection	Plasma hemoglobin	Total hemoglobin in urine	Per cent of injected hemoglobin appearing in urine
		<i>mg. per cent</i>	<i>gm.</i>	
10 gm. in 250 cc.	30 min.	107		
	90 "	92		
	210 "	42	0.850	8.5
	330 "	27	0.025	0.25
	18 hrs.	5	0	
4 " " 100 "	5 min.	94		
	30 "	78	0.30	7.5
	100 "	47		
	180 "	27	0.01	0.25

TABLE V

*Comparison of Relative Absorption Intensity of Two Hemochromogens*

Deuterohemochromogen			Protohemochromogen		
Deuterohemin		Galvanometer reading	Protohemin		Galvanometer reading
<i>mg.</i>	<i>mμ × 10<sup>-3</sup></i>		<i>mg.</i>	<i>mμ × 10<sup>-3</sup></i>	
0.03160	5.25	82.00	0.0227	3.5	83.25
0.06320	10.50	63.50	0.0454	7.0	69.75
0.09470	15.75	49.50	0.0682	10.5	56.00
0.12630	21.0	40.00	0.0909	14.0	48.75
0.15790	26.20	33.50	0.1363	21.0	33.00

of the added hemoglobin was but 77 per cent, there was no increase in fluorescence, indicating that at least no appreciable conversion to porphyrin had occurred.

From a theoretical standpoint the method would be of value in the determination of the fate of ingested hemoglobin or other heme compounds. The method is of practical value for determination of blood loss in the feces, whatever the source of bleeding. It is often extremely difficult, either by inspection alone, or with the aid of the guaiacum and

benzidine tests, to know just how much blood the patient is losing from day to day. It is frequently difficult to be certain that gastrointestinal bleeding is sufficient to account for the degree of anemia observed. It would often be helpful to be able to know the loss of hemoglobin in the feces *per diem*. This is frequently much greater or much less than inspection would indicate. It is of interest that a patient with a bleeding peptic ulcer lost 27 gm. of hemoglobin in a 150 gm. stool specimen (equal to more than 200 cc. of his blood). A patient with chronic ulcerative colitis and mild anemia (12 gm. per cent of hemoglobin) lost 0.2 to 0.8 gm. of hemoglobin in her feces daily for a period of 2 weeks.

*Urine*—Experiments have repeatedly resulted in 95 to 98 per cent recovery of hemoglobin added to the urine. Duplicate determinations

TABLE VI  
*Recovery of Hemoglobin Added to Feces*

Amount of feces	Amount of hemoglobin added	Amount recovered
gm.	mg.	gm.
5	114.0	112.0
5	45.0	40.0
4	53.0	42.5
3	56.0	54.0
10	2.17	2.5

have checked satisfactorily. It is believed that the investigation of any case of hemoglobinuria would be facilitated by such a quantitative method.

#### SUMMARY

A method for the quantitative determination of hemoglobin and heme pigments in plasma, serum, urine, and feces is described. The principle of the method is the conversion to pyridine ferrohemochromogen, followed by colorimetric determination with the Evelyn photoelectric colorimeter. The center setting is determined with a blank solution in which the hemochromogen absorption has been eliminated by means of hydrogen peroxide. The error in recovery is within 10 per cent for urine and plasma. Experiments on various samples of feces have resulted in 75 to 95 per cent recovery. Any fraction of the hemoglobin converted to proto- or deuteroporphyrin is not included. The maximum error that might be introduced by conversion to deuterohematin rather than protohematin is 20 per cent. Theoretical and practical applications of the method are discussed.

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# THE DETERMINATION OF CREATININE AND CREATINE IN BLOOD AND URINE WITH THE PHOTOELECTRIC COLORIMETER

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In spite of their biological importance, measurement of creatinine and creatine in blood plasma has been uncertain because of the low concentrations in which these substances, especially creatine, appear, and the non-specificity of the Jaffe reaction commonly employed for their determination. The presence of creatine in plasma has been questioned since the work of Wilson and Plass (1), because the increment of color after hydrolysis is equivocal when analyzed in visual colorimeters. For this reason Wu (2) concluded that the blood of normal adult males contains no creatine. It seemed possible that by the use of the photoelectric colorimeter, which is peculiarly adapted to the measurement of small changes in the color of faintly tinted solutions, greater accuracy in the measurement of creatinine might be attained and, thereby, that the presence of creatine might be detected and its concentration measured. Such a procedure has been devised and the presence, in appreciable and measurable concentration, of material that is presumably creatine has been established. The procedure is an adaptation of the Folin and Wu (3) colorimetric technique to the Evelyn-Malloy (4) photoelectric colorimeter.

## EXPERIMENTAL

A procedure based on these principles has been employed by Horvath (5) and another is described by the manufacturers of the photoelectric colorimeter.<sup>1</sup> For the calculation of the results the manufacturer proposes the formula  $x = 1000L/K_2$ , where  $K_2 = 6.9$  and  $L = 2 - \log C$ , in which  $C$  represents the galvanometer reading and  $x$ , the creatinine in mg. per cent. The curve obtained by this formula did not, however, agree with the results obtained from the analysis of standard solutions of creatinine and creatine. This is shown in Fig. 1.

For purposes of standardization solutions of creatinine zinc chloride and creatine were used. Both were checked by analysis for nitrogen by the macro-Kjeldahl procedure and in addition it was ascertained that after

<sup>1</sup> Rubicon Company, Philadelphia.

hydrolysis the color reaction given by creatine was equal to that given by an equivalent amount of creatinine zinc chloride which had been subjected to identical treatment, including the hydrolysis. Hydrolysis was effected by autoclaving the solutions at 15 pounds pressure for 20 minutes.

In the original Folin and Wu technique a saturated solution of picric acid was used. In some earlier studies Welt<sup>2</sup> found that it was impossible to duplicate in summer the curves he had defined in the winter with standard creatinine solutions. This he attributed to the fact that the solubility of picric acid varied greatly with temperature (a saturated solution contains 12.2 gm. per liter at 20°, 63.3 gm. at 100°). This was verified in the present

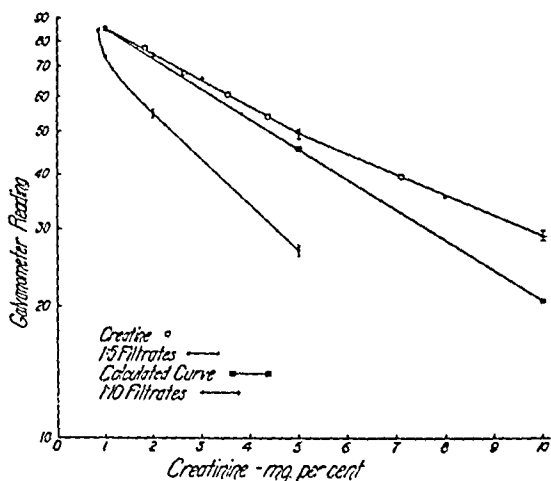


FIG. 1. Curves showing results of analysis of 1:10 and 1:5 tungstic acid filtrates of standard creatinine and creatine solutions and of aqueous solutions of creatinine as well as the calculated curve derived from the formula  $x = 100 (2 - \log 6)/6.9$ . Creatine values are expressed in terms of creatinine.

study. Therefore, instead of a saturated solution, a solution containing 11.75 gm. of picric acid per liter was adopted. This is just sufficiently unsaturated to avoid any danger of precipitation at ordinary laboratory temperatures. It is advisable to keep the solution stoppered in a comparatively cool place to prevent evaporation.

With this modification it proved feasible to measure creatinine in serum with ease and accuracy. By means of standard solutions of creatinine zinc chloride, a curve was drawn which is depicted in the top curve of Fig. 1. This curve has been repeatedly checked for 4 years upon one colorimeter by means of standard solutions and has proved constant.

<sup>2</sup> Welt, L. G., unpublished studies (1937).

On the other hand, it proved inapplicable to a second instrument, ostensibly identical. It is, therefore, necessary to construct a calibration curve for each instrument. It will be noted that the curve plotted on semilogarithmic paper is rectilinear within the limits of accuracy of the procedure with concentrations of creatinine only up to 5 mg. per cent. Above this it diverges perceptibly from linearity. This divergence has been repeatedly verified on two instruments. The reason for the curvature can only be conjectured. It is suspected that it is referable to the bicolorimetric character of the determination (as the color produced by creatinine increases, the color from the alkaline picrate decreases) and the fact that the spectral interval of the filter does not permit precise enough discrimination between the two colors. This curvature limits readings to the range of 1 to 5 mg. per cent in 1:10 filtrates. This spans, however, the most accurate reading range of the colorimeter.

In the original technique of Folin and Wu, for creatine, since hydrochloric acid is added as a dehydrating agent, readings are finally made in a 1:20 dilution, only one-half as concentrated as that used for creatinine. Since normal serum may contain as little as 1 mg. per cent of creatinine and only a fraction of 1 mg. per cent of creatine, it is obvious from the calibration curve of Fig. 1 that this would bring the readings for creatinine and creatine into the range in which the colorimeter is insensitive. With 1:10 filtrates creatinine can be read accurately only in concentrations of 1 to 5 mg. per cent. An attempt was, therefore, made to use 1:5 tungstic acid filtrates in which the ratio of serum to tungstate was the same as it was in the conventional 1:10 filtrates. The lowest curve in Fig. 1 describes the reading on 1:5 filtrates. It will be seen that the object was not achieved by this method, since readings can be made accurately only in the range of 2 to 5 mg. per cent. This would be equivalent to 1 to 2.5 mg. per cent in 1:10 filtrates. It will be noted that the curve is again rectilinear only over this range. It was established that the concentrations of tungstic acid could be varied 100 per cent without affecting the color developed in the Jaffe reaction. It is, therefore, permissible, if the concentration of creatinine in serum is greater than 5 mg. per cent, to double the dilution with water at any stage of the procedure to bring the colorimetric readings within the practicable range of the instrument.

In an effort to discover whether the tungstic acid had any effect on color development, blanks and standards made up in solutions of tungstate of the concentrations found in plasma filtrates were compared with blanks and standards made up in distilled water. In the creatinine procedure tungstate proved to have no effect on the intensity of the color developed. When the same test was applied to the creatine procedure, a precipitate formed during autoclaving in all tungstate filtrates to which hydrochloric



acid was added. This was insoluble in alkaline picrate, rendering readings meaningless, owing to interference with the transmission of light by the precipitate. No such precipitate formed in filtrates of plasma subjected to the same procedure, presumably because the plasma contained sufficient buffer to modify the effect of the acid. To obviate this difficulty smaller amounts of acid were added. This led to the discovery that at autoclave temperatures, *in tungstate filtrates*, creatine was dehydrated completely without the addition of acid. This simplified the procedure and at the same time eliminates the necessity of determining creatine and creatinine in different dilutions. The completeness of dehydration and the accuracy with which creatine can be determined are both illustrated in Fig. 1, in which the circles representing measurements of creatine fall sharply on the 1:10 creatinine curve. It is also evident from this coincidence that the color produced by creatinine is not modified by autoclaving.

### Method

The analytical procedure finally adopted is essentially that of Folin and Wu (3) adapted to the Evelyn colorimeter. Only two important modifications have been introduced: the use of a more dilute picric acid solution and the omission of hydrochloric acid for the dehydration of creatine. The same method has been adapted to the analysis of urine.

*Preparation of Urine*—Folin's (6) original methods for the determination of urinary creatinine and creatine were designed to take advantage of the high concentrations of these compounds in urine and the consequently deeper colors they yielded with alkaline picrate. Such deep colors are, however, unsuited to the photoelectric colorimeter. In attempts to apply the Evelyn instrument to the original technique it was found that standard creatinine solutions yielded inconsistent results. As the photoelectric colorimeter is most accurate with low concentrations of color, and because of the obvious advantages of using the same technique for both blood and urine, the method evolved for analysis of serum has been applied directly to urine.

The variability of concentrations of creatinine and creatine in urine presents obvious difficulties, since the range in which colors can be read is so restricted. This obstacle was surmounted by Tierney<sup>3</sup> by the expedient of diluting all urines to the same specific gravity. The following procedure has, in the great majority of instances, yielded concentrations of creatinine and creatine within the limits which can be read with the colorimeter.

The urine, whether a 24 hour or a fractional specimen, is first measured (V), then diluted to a specific gravity of 1.010, and again measured (V<sub>d</sub>).

<sup>3</sup> Tierney, N. A., to be published.

Of the diluted urine, 5 cc. are again diluted to 100 cc. in a volumetric flask. If the specific gravity of the original urine was less than 1.010, the first dilution is omitted and an aliquot appropriately greater than 5 cc. is diluted to 100 cc. in the volumetric flask. The urine thus finally diluted is analyzed by the procedure described below.

*Reagents—*

1. An N/12 sulfuric acid solution.
2. A 10 per cent solution of sodium tungstate.
3. A picric acid solution, each liter of which contains 11.75 gm. of picric acid which has been purified according to the directions of Folin and Wu (3).
4. A carbonate-free 10 per cent solution of sodium hydroxide.

*Analytical Procedure—*For duplicate measurements of both creatine and creatinine, 5 cc. of serum or of the diluted urine are placed in a flask, followed by 40 cc. of N/12 sulfuric acid and 5 cc. of sodium tungstate solution. After thorough shaking the mixture is filtered (in urines containing no protein in which no precipitate appears filtration may be omitted). Of the protein-free tungstic acid filtrate, 8 cc. are measured into each of four of the special colorimeter tubes, while 8 cc. of water are measured into a fifth tube to serve as a blank. The mouths of two of the four tubes containing filtrate are covered with tin-foil and the tubes are autoclaved at 15 pounds pressure (115–120°) for 20 minutes. At the end of this period the tubes are removed from the autoclave and allowed to cool. The alkaline picrate solution is then prepared by adding 1 volume of 10 per cent sodium hydroxide to 5 parts of the picric acid solution. The alkaline picrate solution should be made up not longer than 5 minutes before it is to be used, as a flaky precipitate forms in it rather rapidly. Although this dissolves readily when the picrate is added to the tungstate filtrate, it makes proper measurement of the solution difficult.

To the contents of each of the five tubes (the two which have been autoclaved, the two containing unautoclaved filtrate, and the water blank) are added 4 cc. of the alkaline picrate solution. The tubes are set aside for 20 minutes to permit complete development of color, before colorimetric readings are made. Readings need not be made immediately after this interval, however, as the color remains unchanged for at least 2½ hours. After the color has been developed, the readings are made in the colorimeter with a 520  $\mu$  filter (transmission 495 to 550  $\mu$ ; Rubicon glasses Nos. 3522, 4306, and 5032). The blank tube is first introduced and, with this in place, the galvanometer is set at 100. The colors of the other tubes are then read in the usual manner.

*Calculations—*The galvanometer readings are converted to terms of creatinine by interpolation on a curve constructed by plotting readings

obtained from standard solutions of creatinine zinc chloride (7). Reagents should be checked from time to time by analyses of standard solutions. If creatine solutions are employed, the determinations must be made on the day the solutions are prepared, because creatine solutions are quite unstable. The curve for creatinine is conveniently graduated in terms of mg. per cent of creatinine in the original specimen. The difference between the readings of the autoclaved and unautoclaved specimens represents creatine in terms of creatinine. This is converted to creatine by multiplying by the factor 1.16.

TABLE I  
*Creatinine and Creatine Added to Serum*

Added		Recovery		Error		Total error	
Creatinine (1.35 mg. per cent originally)	Creatine* (0.89 mg. per cent originally)	Creatinine	Creatine†	Creatinine	Creatine	Creatinine	Creatine
mg. per cent	mg. per cent	mg. per cent	mg. per cent	per cent	per cent	per cent	per cent
2	0	3.25	4.39	-3.0	+17	-3.0	+3.5
0	1.9	1.50	3.95	+7.5	-7	+7.5	-4.6

\* Expressed as creatinine.

† Expressed as creatine + creatinine.

The following formulas may be used to calculate the concentrations and amounts of creatinine and creatine in urine,

$$\frac{V_d \times 100 \times C}{V \times a} = X = \text{mg. creatinine in 100 cc. of original specimen}$$

$$\frac{V \times X}{100} = \text{gm. creatinine in total specimen of urine}$$

where  $a$  = the aliquot of the diluted urine, in cc.;  $V$  = the volume of the undiluted urine in liters; and  $V_d$  = the volume to which the urine was diluted to bring its specific gravity to 1.010.

If the reading of creatinine (or creatine + creatinine, expressed in terms of creatinine) on the curve exceeds 5 mg. per cent, in the analysis of plasma or urine either a smaller aliquot must be taken or the tungstic acid filtrate must be diluted with water before analysis. Ordinary 1:10 or even 1:5 filtrates, diluted with equal volumes of water, give readings that are proportional to the dilution.

The total error of colorimeter readings, as judged by the analysis of standard solutions of creatinine and of creatine + creatinine, is slightly less than 4 per cent over the range of 1 to 5 mg. per cent, as is evident from

the data represented in Fig. 1. The error in measurement of creatine added to creatinine is somewhat greater, amounting at times to as much as 10 per cent, because, since creatine is measured by difference, it incurs a double error. Creatinine added to human sera may be recovered quantitatively. The instability of creatine renders its recovery somewhat less reliable. However, in analyses after such additions, at least 80 per cent of creatine was recovered as creatine, and at least 95 per cent as creatinine + creatine. An example of such an analysis is given in Table I. The error, within concentrations thus far encountered in serum, does not exceed 0.1 mg. per cent.

A series of analyses of individual and pooled human sera gave values of 0.9 to 1.7 mg. per cent for creatinine. A small series of analyses of normal human sera gave values averaging 0.3 mg. per cent for creatine. In pooled sera from the clinical laboratory as much as 1.1 mg. per cent was found. Analyses of urine of normal adult males yielded values for creatinine within the generally accepted limit. No creatine could be detected in such urines, although it was found in certain pathological urines. This is at variance with the reports of Dill and Horvath (8).

The method has been employed for a year in the chemical laboratory of the Department of Internal Medicine of the Yale University School of Medicine and has given consistent results.<sup>3</sup>

#### DISCUSSION

Though the presence of creatinine in the blood serum of humans had been definitely established for some time (9-12), creatine was present, if at all, in such low concentrations that its determination was impossible by optical colorimetry. Therefore, the availability of a method sufficiently sensitive to detect the presence of definite quantities of "apparent creatine" should open up avenues of exploration in a hitherto inaccessible field. Since a large percentage of the chromogens present in the cells of blood is presumably not creatinine (11), no attempts have been made as yet to apply the method to whole blood. At the present time definite evidence that the serum chromogens obtained by hydrolysis of tungstate filtrates are actually creatine is lacking, and the application of enzymatic methods to blood such as have been employed in tissues (13) is to be desired. However, presumptive confirmatory evidence that the "apparent creatine" found in the serum by this method actually is creatine has been recently furnished in this laboratory by the discovery that its concentration in persons with creatinuria is definitely elevated.<sup>3</sup>

The urine method presented here does not represent a theoretical advance from previous procedures employing optical colorimeters (6), but does possess the advantage of simplicity and somewhat increased accuracy.

## SUMMARY

The Jaffe reaction for the determination of serum creatinine is adapted for use with the Evelyn-Malloy photoelectric colorimeter, with a constant strength of picric acid solution.

In the measurement of creatine, conversion to creatinine is accomplished by autoclaving without the use of hydrochloric acid acidification.

The method can be applied to the analysis of urines, with the same reagents and calibration curve that are used in the analysis of serum.

The total colorimetric error is 4 per cent of theoretical.

Creatinine added to serum is recovered quantitatively.

0.9 to 1.7 mg. per cent of creatinine was found in normal and pooled human sera.

Definite amounts of creatine, averaging 0.3 mg. per cent, were found in normal human sera.

The amounts of creatinine and creatine found in urine were consistent with the findings of other observers.

The author wishes to express his indebtedness to Miss Pauline M. Hald whose advice and assistance have proved invaluable.

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# THERMODYNAMIC PROPERTIES OF SOLUTIONS OF AMINO ACIDS AND RELATED SUBSTANCES

## VIII. THE IONIZATION OF GLYCYLGLYCINE, $\epsilon$ -AMINOCAPROIC ACID, AND ASPARTIC ACID IN AQUEOUS SOLUTION FROM ONE TO FIFTY DEGREES\*

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The effect of temperature on the thermodynamic ionization constants of compounds with dipole moments greater than those of the  $\alpha$ -amino acids has not heretofore been studied. In this paper results are given for glycylglycine and  $\epsilon$ -aminocaproic acid, two substances with similar dipole moments. Measurements are also included for aspartic acid, which has recently been studied at 25° in cells without liquid junction (1).

### Methods and Materials

The glycylglycine and  $\epsilon$ -aminocaproic acid were from samples used in earlier studies (11).

The *dl*-aspartic acid was purchased from Amino Acid Manufactures. It was recrystallized twice from hot water and dried over phosphorus pentoxide.

The preparation of the hydrochloric acid, sodium hydroxide, and sodium chloride solutions is described in an earlier paper (12) as is the preparation of the electrodes (13).

### RESULTS AND DISCUSSION

The equations for calculating the ionization constants of glycylglycine and  $\epsilon$ -aminocaproic acid are the same as those employed for the other amino acids (7, 12, 13).

The electromotive force of a buffer solution of a monoaminomonocarboxylic acid and hydrochloric acid is related to the first acid ionization constant by the equation

$$\begin{aligned} \text{p}K_1 - \log \frac{\gamma_{\text{HAH}^+}}{\gamma_{\text{H}^+} \gamma_{\text{HA}^{\pm}}} \\ = \frac{(E - E_0)nF}{2.3026RT} + \log m_{\text{Cl}^-} + \log \gamma_{\text{H}^+} \gamma_{\text{Cl}^-} + \log \frac{m_{\text{Cl}^-} - m_{\text{H}^+}}{m_{\text{A}} - m_{\text{Cl}^-} + m_{\text{H}^+}} \quad (1) \end{aligned}$$

\* Aided by a grant from the Fluid Research Fund of the Yale University School of Medicine.

Buffer solutions of amino acid, sodium hydroxide, and sodium chloride yield electromotive force measurements related to the second acid ionization constant by the equation

$$pK_2 + \log \frac{\gamma_{A^-}}{\gamma_{HA} \pm \gamma_{Cl^-}} = \frac{(E - E_0)nF}{2.3026RT} + \log m_{Cl^-} + \log \frac{m_A - m_{NaOH} + m_{OH^-}}{m_{NaOH} - m_{OH^-}} \quad (2)$$

The equations necessary for the calculation of the aspartic acid constants are more complicated, since the first two acid constants are sufficiently close together so that the effect of one on the other cannot be neglected. For the first acid constant, from the equation representing the total amino acid,  $T_A$ , as being present in solution in four possible forms,

$$T_A = m_{H_2AH^+} + m_{HAH^\pm} + m_{HA^-} + m_{A^-} \quad (3)$$

the equation for electroneutrality

$$m_{H_2AH^+} + m_{H^+} = m_{Cl^-} + m_{HA^-} + m_{OH^-} \quad (4)$$

and the equilibrium constant

$$K_1 = \frac{m_{H^+} m_{HAH^\pm}}{m_{H_2AH^+}} \frac{\gamma_{H^+} \gamma_{HAH^\pm}}{\gamma_{H_2AH^+}} \quad (5)$$

the following equation may be derived, since the concentrations of  $m_{A^-}$  and of  $m_{OH^-}$  may be neglected in the acid solutions.

$$m_{H^+} = K_1 \frac{m_{Cl^-} - m_{H^+} + m_{HA^-}}{T_A - m_{Cl^-} + m_{H^+} - 2m_{HA^-}} \frac{\gamma_{H_2AH^+}}{\gamma_{H^+} \gamma_{HAH^\pm}} \quad (6)$$

When this equation for the hydrogen ion concentration is combined with that for the E.M.F. of the cell, in which the experimental data may be directly substituted,

$$-\log m_{H^+} = \frac{(E - E_0)nF}{2.3026RT} + \log m_{Cl^-} + \log \gamma_{H^+} \gamma_{Cl^-} \quad (7)$$

the following useful equation may be derived.

$$pK_1 - \log \frac{\gamma_{H_2AH^+}}{\gamma_{H^+} \gamma_{HAH^\pm}} = \frac{(E - E_0)nF}{2.3026RT} + \log m_{Cl^-} + \log \gamma_{H^+} \gamma_{Cl^-} + \log \frac{m_{Cl^-} - m_{H^+} + m_{HA^-}}{T_A - m_{Cl^-} + m_{H^+} - 2m_{HA^-}} \quad (8)$$

Of the unknown values on the right-hand side of Equation 8 those for  $\log \gamma_{H^+} \gamma_{Cl^-}$  may be obtained from the data of Harned and Ehlers (4). By assuming an approximate value for  $K_2$ , values of  $m_{HR^-}$  may be obtained from Equations 3 and 4 and Equation 10 (in which activities are neglected) combined to give

$$m_{HA^-} = \frac{K'_2(T_A - m_{Cl^-} + m_{H^+})}{m_{H^+} + 2K_2} \quad (9)$$

By combining the equation for the second acid constant

$$K_2 = \frac{m_{H^+} \cdot m_{HA^-}}{m_{HAH^+}} \frac{\gamma_{H^+} \gamma_{HA^-}}{\gamma_{HAH^+}} \quad (10)$$

with that representing the total amino acid in all its forms, Equation 3, and that for electroneutrality

$$m_{H_2AH^+} + m_{H^+} + m_{Na^+} = m_{HR^-} + m_{OH^-} \quad (11)$$

an equation for the hydrogen ion concentration is obtained

$$m_{H^+} = K_2 \frac{T_A - m_{Na^+} - m_{H^+} - 2m_{H_2AH^+}}{m_{H_2AH^+} + m_{NaOH} + m_{H^+}} \frac{\gamma_{HAH^+}}{\gamma_{H^+} \gamma_{HA^-}} \quad (12)$$

in which the concentrations of  $m_{A^-}$  and  $m_{OH^-}$  may be neglected. The value for  $m_{Na^+}$  is equivalent to that of  $m_{NaOH}$ , since the  $m_{Na^+}$  from the sodium chloride may be canceled by the equivalent  $m_{Cl^-}$ . By combining Equation 12 with the other equation for the hydrogen ion concentration (Equation 7) and placing the results in logarithmic form, Equation 13 is obtained.

$$\begin{aligned} pK_2 - \log \frac{\gamma_{HAH^+}}{\gamma_{H^+} \gamma_{HA^-}} &= \frac{(E - E_0)nF}{2.3026RT} + \log m_{Cl^-} \\ &+ \log \gamma_{H^+} \gamma_{Cl^-} + \log \frac{T_A - m_{NaOH} - m_{H^+} - 2m_{H_2AH^+}}{m_{NaOH} + m_{H^+} + m_{H_2AH^+}} \end{aligned} \quad (13)$$

The equation for calculating  $m_{H_2AH^+}$  may be obtained by combining Equation 5 with Equations 3 and 11 to give

$$m_{H_2AH^+} = \frac{m_{H^+}(T_A - m_{H^+} - m_{NaOH})}{K_1 + 2m_{H^+}} \quad (14)$$

The equation for the third constant

$$K_3 = \frac{m_{H^+} m_{A^-}}{m_{HA^-}} \frac{\gamma_{H^+} \gamma_{A^-}}{\gamma_{HA^-}} \quad (15)$$

can be combined with Equation 3, in which  $m_{H_2AH^+}$  and  $m_{HAH^+}$  may be neglected in alkaline solutions, and the equation for electroneutrality,

$$m_{Na^+} + m_{H^+} = m_{HA^-} + m_{OH^-} + 2m_{A^-} \quad (16)$$

in which the molality of the hydrogen ion may be neglected, as the solutions are alkaline and  $m_{Na^+}$  represents the molality of the sodium from the sodium hydroxide, to give an equation for the third constant

$$K_3 = \frac{m_{H^+}(m_{NaOH} - T_A - m_{OH^-})}{2T_A - m_{NaOH} + m_{OH^-}} \frac{\gamma_{H^+} \gamma_{A^-}}{\gamma_{HA^-}} \quad (17)$$



which may be placed in logarithmic form and combined with Equation 7 to give Equation 18.

$$\text{pK}_a = \frac{(E - E_0)nF}{2.3026RT} + \log m_{\text{Cl}^-} + \log \frac{2T_A - m_{\text{NaOH}} + m_{\text{OH}^-}}{m_{\text{NaOH}} - T_A - m_{\text{OH}^-}} + \log \frac{\gamma_{\text{H}^+} \gamma_{\text{Cl}^-} \gamma_{\text{HA}^-}}{\gamma_{\text{H}^+} \gamma_{\text{A}^-}} \quad (18)$$

TABLE I

*Experimental Data Used in Determining First Acid Ionization Constants of Glycylglycine,  $\epsilon$ -Aminocaproic Acid, and Aspartic Acid*

Molality		Observed $E$ , corrected to 1 atmosphere $\text{H}_2$ , at				
Experimental substance	HCl	1.0°	12.5°	25.0°	37.5°	50.0°
Glycylglycine						
0.02883	0.01835	0.5004	0.5038	0.5075	0.5111	0.5140
0.03089	0.01823	0.5042	0.5083	0.5118	0.5155	0.5177
0.03156	0.01952	0.5005	0.5037	0.5076	0.5109	0.5136
0.03919	0.02198	0.5024	0.5058	0.5096	0.5135	0.5165
0.04540	0.02792	0.4926	0.4949	0.4983	0.5016	0.5038
0.06146	0.03209	0.4969	0.4999	0.5037	0.5075	0.5103
0.08803	0.04774	0.4870	0.4895	0.4929	0.4957	0.4991
$\epsilon$ -Aminocaproic acid						
0.03200	0.01781	0.5719	0.5785	0.5852	0.5927	0.6003
0.03882	0.02259	0.5641	0.5707	0.5770	0.5844	0.5918
0.04830	0.02700	0.5638	0.5689	0.5755	0.5828	0.5900
0.05862	0.03305	0.5587	0.5643	0.5705	0.5776	0.5846
0.08642	0.04853	0.5512	0.5557	0.5619	0.5686	0.5752
Aspartic acid						
0.03138	0.01776	0.4598	0.4616	0.4625	0.4627	0.4620
0.02956	0.01839	0.4572	0.4586	0.4592	0.4594	0.4581
0.04066	0.02091	0.4577	0.4585	0.4592	0.4593	0.4584
0.03904	0.02244	0.4528	0.4538	0.4544	0.4540	0.4528
0.04676	0.02609	0.4492	0.4498	0.4500	0.4490	0.4501
0.05906	0.03233	0.4432	0.4436	0.4436	0.4428	0.4416
0.07689	0.04145	0.4371	0.4370	0.4367	0.4348	0.4306

In the last term of Equation 18, the logarithm of the activity coefficient of each ion may be represented by the Debye-Hückel term  $-Az^2\sqrt{\mu} \pm f(\mu)$  in which  $A$  is the theoretical Debye-Hückel constant,  $z$  the valence of the ion, and  $f(\mu)$  denotes a function of the ionic strength that need not be

evaluated. If these equivalents are substituted in Equation 18, the final term becomes  $2A\sqrt{\mu} \pm f(\mu)$ . This may be used in evaluating the equa-

TABLE II

*Experimental Data Used in Determining Second Acid Ionization Constants of Glycylglycine,  $\epsilon$ -Aminocaproic Acid, and Aspartic Acid and Third Acid Ionization Constants of Aspartic Acid*

Molality			Observed $E$ , corrected to 1 atmosphere $H_2$ , at				
Experimental substance	NaOH	NaCl	1.0°	12.5°	25.0°	37.5°	50.0°
Glycylglycine							
0.01485	0.00838	0.00773	0.8437	0.8429	0.8422	0.8410	0.8389
0.01941	0.01083	0.01000	0.8369	0.8366	0.8353	0.8338	0.8314
0.02396	0.01289	0.01233	0.8302	0.8290	0.8275	0.8254	0.8224
0.03146	0.01627	0.01663	0.8210	0.8196	0.8175	0.8149	0.8117
0.04418	0.02333	0.02241	0.8150	0.8134	0.8110	0.8085	0.8052
$\epsilon$ -Aminocaproic acid							
0.01662	0.00847	0.00808	0.9826	0.9831	0.9829	0.9819	0.9798
0.02006	0.01044	0.00994	0.9790	0.9895	0.9792	0.9779	0.9762
0.03172	0.01690	0.01540	0.9705	0.9714	0.9705	0.9688	0.9666
0.04404	0.02400	0.02189	0.9642	0.9642	0.9635	0.9620	0.9597
Aspartic acid, $pK_2$							
0.01980	0.00977	0.00914	0.5645	0.5691	0.5739	0.5786	0.5837
0.02467	0.01192	0.01146	0.5589	0.5632	0.5677	0.5722	0.5767
0.02685	0.01268	0.01125	0.5586	0.5624	0.5671	0.5714	0.5759
0.03029	0.01669	0.01571	0.5579	0.5617	0.5660	0.5706	0.5753
0.04476	0.02373	0.02093	0.5487	0.5520	0.5559	0.5594	0.5639
Aspartic acid, $pK_1$							
0.01560	0.02377	0.00647	0.9268	0.9320	0.9373	0.9420	0.9465
0.01560	0.02356	0.00650	0.9259	0.9309	0.9360	0.9406	0.9447
0.01779	0.02474	0.00703	0.9131	0.9178	0.9221	0.9261	0.9297
0.02134	0.03066	0.00854	0.9119	0.9173	0.9219	0.9257	0.9295
0.02045	0.03185	0.00735	0.9267	0.9319	0.9373	0.9420	0.9461
0.02488	0.03592	0.01040	0.9076	0.9119	0.9163	0.9201	0.9239
0.03116	0.04769	0.01150	0.9131	0.9175	0.9221	0.9260	0.9297
0.04469	0.07016	0.01162	0.9161	0.9205	0.9248	0.9284	0.9315

tion, but the slope is much greater than if values for  $-\log \gamma_{H^+} \gamma_{Cl^-}$  are used (see Equation 15).

The compositions of the buffer solutions and the observed potentials

are recorded on Tables I and II. Fig. 1 illustrates the plot at 25° of the right-hand sides of the final equations for  $pK_1$ ,  $pK_2$ , and  $pK_3$  (Equations 1, 2, 8, 13, and 18) against the ionic strength  $\mu$ . For  $pK_3$  of aspartic acid plots are given with both  $2A\mu$  and  $-\log \gamma_H + \gamma_{Cl^-}$  as the final term of Equation 16. The intercepts at 0 ionic strength are  $pK_1$ ,  $pK_2$ , and  $pK_3$ , respectively. A summary of these values appears in Tables III and IV.

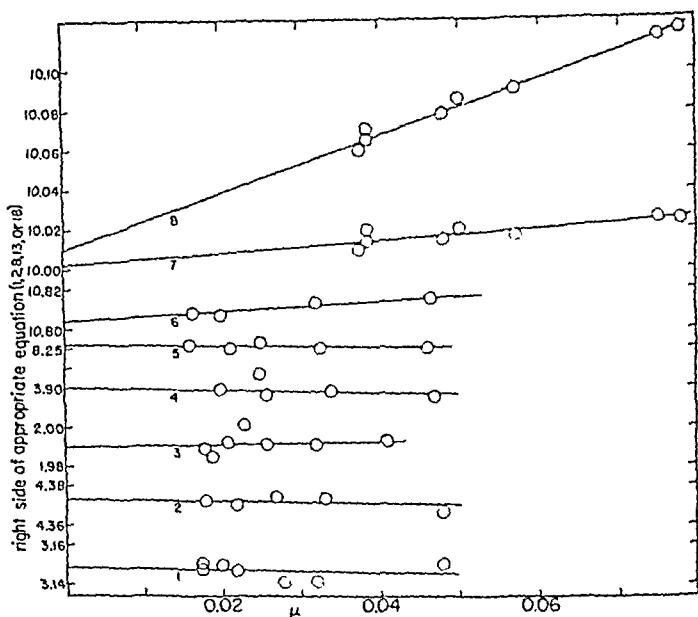


FIG. 1. A plot of the right-hand side of the appropriate equation against the ionic strength. The intercept at 0 ionic strength is  $pK_1$ ,  $pK_2$ , or  $pK_3$ . Curve 1 represents  $pK_1$  for glycylglycine; Curve 2,  $pK_1$  for  $\epsilon$ -aminocaproic acid; Curve 3,  $pK_1$  for aspartic acid; Curve 4,  $pK_2$  for aspartic acid; Curve 5,  $pK_2$  for glycylglycine; Curve 6,  $pK_2$  for  $\epsilon$ -aminocaproic acid; Curve 7,  $pK_3$  for aspartic acid with  $-\log \gamma_H + \gamma_{Cl^-}$  as the final term of Equation 18; Curve 8,  $pK_3$  for aspartic acid with  $2A\sqrt{\mu}$  as the final term of Equation 18.

The values for  $pK_3$  of aspartic acid are from Equation 18 with values for  $-\log \gamma_H + \gamma_{Cl^-}$  in pure solution. The values for the other thermodynamic functions are calculated by the equation of Harned and Embree (5). The results are presented in Tables III to V.

The data at 25° for glycylglycine ( $pK_1$  3.15,  $pK_2$  8.25) are similar to the  $pK'$  values found by Simms (10) ( $pK'_1$  3.12,  $pK'_2$  8.07) and Neuberger (6) ( $pK'_1$  3.08,  $pK'_2$  8.26). The values for  $pK_1$  and  $pK_2$  of  $\epsilon$ -aminocaproic acid are similar to those found by Edsall and Blanchard (3) ( $pK'_1$  4.43,  $pK'_2$

10.75) and are not far from those of a simple aliphatic acid and of an amine respectively. This has been discussed by Cohn (2) who suggested that the proximity of a peptide bond in glycylglycine causes both the  $pK_1$  and  $pK_2$

TABLE III

*Thermodynamic Functions for Ionization of Glycylglycine,  $\epsilon$ -Aminocaproic Acid, and Aspartic Acid*

	1.0°	12.5°	25.0°	37.5°	50.0°
Glycylglycine					
$pK_1$	3.201	3.166	3.148	3.148	3.148
" *	3.201	3.166	3.146	3.141	3.159
$\Delta F_1$	4033	4140	4291	4463	4670
$\Delta H_1$	1190	862	391	-128	-736
$\Delta S_1$	-10.4	-11.5	-12.9	-14.8	-16.7
$\epsilon$ -Aminocaproic acid					
$pK_1$	4.420	4.387	4.373	4.384	4.410
" *	4.404	4.384	4.376	4.384	4.408
$\Delta F_1$	5523	5860	5965	6230	6512
$\Delta H_1$	818	459	-8	-561	-1204
$\Delta S_1$	-17.2	-18.9	-20.0	-21.9	-23.9
Aspartic acid, $pK_1$					
$pK_1$	2.122	2.054	1.990	1.945	1.907
" *	2.128	2.057	1.995	1.948	1.917
$\Delta F_1$	2668	2686	2720	2769	2835
$\Delta H_1$	2324	2094	1783	1373	889
$\Delta S_1$	-1.3	-2.1	-3.1	-4.5	-6.0
Aspartic acid, $pK_2$					
$pK_2$	4.006	3.944	3.900	3.878	3.870
" *	4.004	3.952	3.910	3.883	3.873
$\Delta F_2$	5021	5165	5335	5518	5726
$\Delta H_2$	1761	1448	1110	619	57
$\Delta S_2$	-11.9	-13.0	-14.2	-15.8	-17.5

\* Values calculated from the equation of Harned and Embree (5).

values to be more acid than in a dipolar ion of similar length with only methylene groups between the charged groups. The values at 25° for aspartic acid ( $pK_1$  1.99,  $pK_2$  3.90,  $pK_3$  10.00) may be compared with those of Batchelder and Schmidt (1) ( $pK_1$  2.01,  $pK_2$  3.90,  $pK_3$  9.84). The only appreciable discrepancy is in  $pK_3$ . The values for  $pK_3$  in Fig. 1 have been

obtained by two simple applications of the Debye-Hückel equation. The uncertainty in the extrapolation by the two methods is shown in Fig. 1.

TABLE IV  
Thermodynamic Functions for Ionization of Glycylglycine,  $\epsilon$ -Aminocaproic Acid, and Aspartic Acid

	1.0°	12.5°	25.0°	37.5°	50.0°
Glycylglycine					
pK <sub>2</sub>	8.944	8.594	8.252	7.948	7.668
" *	8.938	8.598	8.254	7.943	7.668
$\Delta F_2$	11,210	11,240	11,260	11,290	11,340
$\Delta H_2$	10,610	10,660	10,600	10,400	10,060
$\Delta S_2$	-2.2	-2.0	-2.0	-2.8	-4.0
$\epsilon$ -Aminocaproic acid					
pK <sub>2</sub>	11.666	11.244	10.804	10.406	10.036
" *	11.662	11.234	10.804	10.403	10.034
$\Delta F_2$	14,645	14,670	14,740	14,790	14,820
$\Delta H_2$	13,120	13,390	13,560	13,620	13,550
$\Delta S_2$	-5.6	-4.5	-4.0	-3.8	-3.9
Aspartic acid, pK <sub>3</sub>					
pK <sub>3</sub>	10.604	10.304	10.002	9.742	9.512
" *	10.596	10.299	10.006	9.744	9.506
$\Delta F_3$	13,280	13,450	13,650	13,850	14,060
$\Delta H_3$	9,280	9,220	9,025	8,695	8,220
$\Delta S_3$	-14.6	-14.8	-15.5	-16.6	-18.1

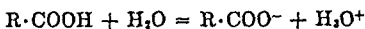
\* Values calculated from the equation of Harned and Embree (5).

TABLE V  
Maxima of Acid Ionization Constants and Temperatures of Maximum Ionization of Glycylglycine,  $\epsilon$ -Aminocaproic Acid, and Aspartic Acid

Substance	pK <sub>1,max.</sub>	$\theta_1$	pK <sub>2,max.</sub>	$\theta_2$	pK <sub>3,max.</sub>	$\theta_3$
Glycylglycine .....	3.141	24.8	6.558	155.3		
$\epsilon$ -Aminocaproic acid...	4.376	68.6	8.024	191.8		
Aspartic acid.....	1.900	52.2	3.873	52.2	8.774	136.0

These values are appreciably higher than Batchelder and Schmidt obtained with a modification of the Debye-Hückel equation suggested by Simms (9). The reason for this difference is not apparent.

Pitzer (8) has pointed out that the ionization of a fatty acid in water



is always accompanied by a large decrease in entropy, about  $-22$  electrostatic units per mole, due chiefly to the reorientation of water molecules around the charged ions. The work on the  $\alpha$ -amino acids (12, 13) showed that  $\Delta S_1$  for these compounds is much lower, about  $-7$  electrostatic units, since the charge on the adjoining  $-\text{NH}_3^+$  group opposes the orientation of water molecules around the  $-\text{COO}^-$  group. The data on  $\epsilon$ -aminocaproic acid give  $\Delta S_1$  near  $-20$  electrostatic units, a decrease almost as great as that found in an unsubstituted fatty acid, showing strikingly the effect of the increased distance between the ammonium and carboxyl group.<sup>1</sup>

#### SUMMARY

The thermodynamic ionization constants of glycylglycine,  $\epsilon$ -aminocaproic acid, and aspartic acid have been determined from  $1$ – $50^\circ$ . The values for the derived thermodynamic functions have been calculated.

We are indebted to Professor D. I. Hitchcock for advice and criticism.

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<sup>1</sup> We are indebted to Dr. John T. Edsall for the suggestion.



# DETERMINATION OF THE OXYGEN CONTENT OF SMALL QUANTITIES OF BODY FLUIDS BY POLAROGRAPHIC ANALYSIS

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Polarographic analysis was originated by Heyrovsky about 20 years ago. It is based upon the observation that when an electrolyte solution is electrolyzed in a cell composed of a dropping mercury electrode and a second non-polarizable electrode a current-voltage curve is obtained which is characteristic of both the nature and concentration of the electrooxidizable or electroreducible substances present. The basic theory and the limitations of the method have been extensively studied by Kolthoff and Lingane and reported in their recent monograph (6). Factors often neglected in quantitative polarography have been discussed recently by Kolthoff (5).

The adaptability of the polarograph<sup>1</sup> to the study of oxygen was utilized by Vitek (11); he found it suitable for determination of the concentration of oxygen in technical gases. Ingols (4) has used the method to measure the oxygen content of activated sludge, and Manning (8) has determined oxygen concentration at various depths in lake water. Petering and Daniels (9) have reported the special adaptability of the technique to the study of photosynthesis and the respiration rates of microorganisms.

Our concern with the polarograph (generally applicable to the determination of concentrations down to  $10^{-6}$  M) has been to apply it to the determination of the oxygen content of small quantities of body fluids, since other existing methods for measuring the quantity of dissolved oxygen are generally unsatisfactory in this case, either because of the small quantities of fluid available or the low concentration of oxygen present (the Van Slyke method often cannot be employed under these circumstances), or due to the complicating presence of organic material (the Winkler method (12) is unsatisfactory in this case), or due to the presence of re-

<sup>1</sup> After the present study had been submitted for publication, our attention was called to a recent article by Berggren (1) in which he has applied two methods to the determination of physically dissolved oxygen in arterial blood, first, an evacuation method, a further development of the technique of Van Slyke and Neill (10), and, second, a polarographic method. He found the error of the polarographic method to be about half the error of the evacuation method.



ducing substances (the Brinkman and van Schreven method (2) is unsatisfactory when these are present).

The polarographic determination of oxygen has several advantages over classical methods, the most important of which are its accuracy at low concentrations and its ready adaptability to small samples.

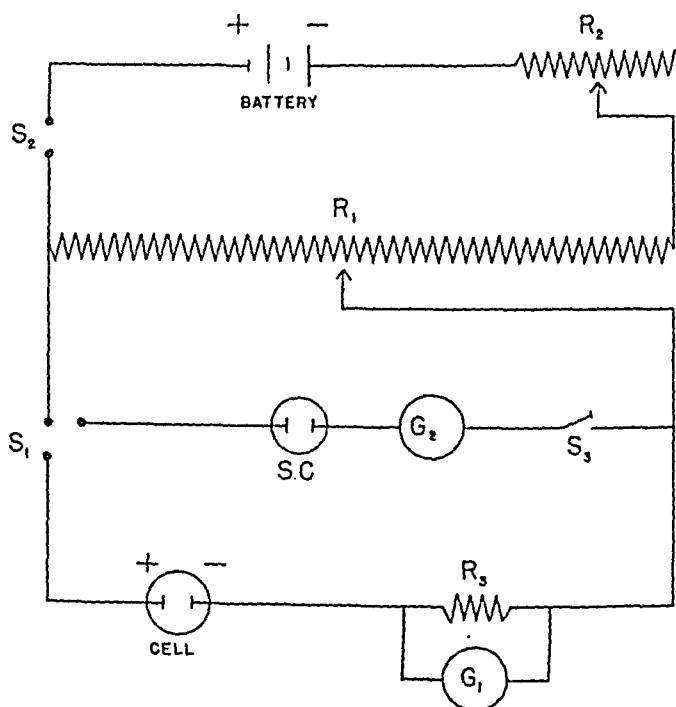


FIG. 1. Line diagram showing the arrangement of the electrical circuit used. *Cell* refers to the dropping mercury electrode cell shown in Fig. 2;  $G_1$ , a Leeds and Northrup galvanometer No. 2284, used for measuring the current produced;  $R_1$ , resistance used to decrease the sensitivity of  $G_1$ ;  $S_3$ , a tapping key;  $G_2$ , a Leeds and Northrup galvanometer No. 2420-C used as a null galvanometer to standardize the potentiometer; *S.C.*, an Eppley standard cell;  $S_1$ , a monopole double throw switch allowing connection of the potentiometer to either the standard cell or the dropping mercury electrode;  $S_2$ , a switch for closing the battery circuit;  $R_1$ , a calibrated resistance which controls the quantity of voltage applied;  $R_2$ , an adjustable resistance used to standardize the potentiometer ( $R_1$  and  $R_2$  constitute a Leeds and Northrup type K potentiometer); *battery*, two No. 6 dry cells used to supply the voltage.

### Methods

A manual type of polarograph was assembled from ordinary laboratory apparatus. Fig. 1 shows the diagram of the electrical circuit. Two dry

cells and a type K potentiometer<sup>2</sup> provide a known variable electromotive force to the cell, while a galvanometer of optimum sensitivity measures the resulting current.

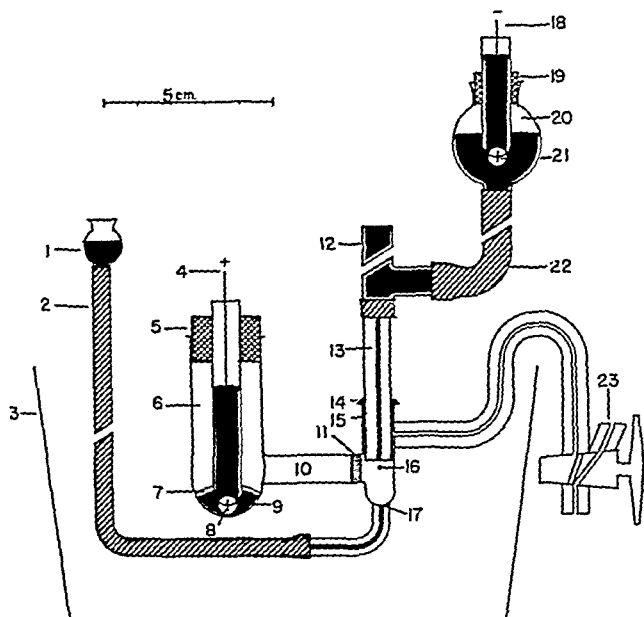


FIG. 2. Arrangement of the dropping mercury cell and external reference electrode. 1, leveling bulb with mercury used for drawing in and expelling the sample analyzed; 2, rubber connecting tube; 3, water bath with thermoregulator; 4, copper wire contact (anode); 5, rubber stopper; 6, saturated potassium chloride; 7, mercurous chloride; 8, platinum wire inserted in the anode tube for connection with the calomel half-cell; 9, mercury; 10, agar-salt bridge; 11, sintered glass disk; 12, 60 cm. glass stand-tube; 13, capillary with 0.05 mm. bore; 14, mercury seal; 15, rubber tubing seal; 16, mercury drop in the reaction chamber; 17, cell chamber ending in a 2 mm. capillary to minimize the area of the interface between the mercury and the solution to be analyzed; 18, copper wire contact (cathode); 19, rubber stopper; 20, leveling bulb with mercury used to maintain a constant pressure on the dropping mercury capillary; 21, platinum wire inserted in the cathode tube for connection with the mercury leading to the capillary; 22, rubber connecting tube; 23, 3-way stop-cock (1 mm. bore).

Fig. 2 shows the arrangement of the cell we have found to be most suitable for the determination of the oxygen content of small quantities of body

<sup>2</sup> If a potentiometer is not available, a simple resistance arrangement is satisfactory.

fluids. The cell is an adaptation of the type employed by Lingane and Laitinen (7). We have arranged the cell for determinations on small quantities of fluid without contact with the air. The inclusion of a suitable reference electrode is of great importance, all too often neglected in polarographic measurement. We have followed the suggestion of Lingane in this regard and have employed an outside standard half-cell, with a saturated calomel electrode. This is also recommended and discussed in a recent paper by Kolthoff (5). The standard half-cell is connected with the electrolysis cell by means of an agar-salt bridge (3 per cent agar in saturated potassium chloride) as shown in Fig. 2. It is replaced after about every five or six experiments. This bridge, of course, adds resistance which has to be considered when the relationship between current and potential is studied (Kolthoff and Lingane (6) p. 137).

The cell is kept in a constant temperature bath, maintained at  $25^{\circ} \pm 0.5^{\circ}$ ; variations in current due to temperature fluctuation are then kept below  $\pm 1$  per cent. The volume of the electrolysis chamber was arbitrarily chosen as approximately 1.0 cc., since this was convenient for our proposed use of the method. This volume can be made larger or probably smaller, depending on the quantity of fluid available.

### *Preparation and Use of Apparatus*

We have so far studied lymph and blood plasma. The method should be applicable to other body fluids, provided they do not contain substances other than oxygen that are reduced at the dropping electrode in such a way as to interfere with the interpretation of the polarogram being studied.

*Galvanometer*—The sensitivity of the galvanometer used should be determined. This can be done as described by Kolthoff and Lingane ((6) p. 228). At maximum sensitivity the galvanometer we used registers 1 mm. of deflection for every  $9.6 \times 10^{-3}$  microampere of current. When the oxygen determinations were made, a resistance shunt was attached to the galvanometer. In this case the galvanometer registered 1 mm. for every  $9.4 \times 10^{-2}$  microampere. The period of the galvanometer with the resistance shunt attached was 15 seconds.

*Capillary*—"Marine barometer tubing" supplied by the Corning Glass Works provides a satisfactory capillary. This tubing has a bore of about 0.05 mm. and an outside diameter of 6.0 mm., and we used it in 6.0 cm. lengths. A constant pressure on the mercury at the capillary orifice was obtained by raising the mercury in the stand-tube (12 in Fig. 2) to the desired level. This height was chosen arbitrarily as one which would provide the optimum rate of mercury drop formation; that is, 1 drop in 3 to 6 seconds. This level was then marked and maintained constant throughout the life of the capillary. With the fluids we have used, it has

been necessary to replace the capillary after every five to seven experiments. Slowing in the rate of the mercury dropping can be corrected for within certain limits by recalibration of the capillary; however, when the rate becomes slower than 1 drop in 6 seconds, the capillary is discarded.

Before and after each use, the capillary tip is cleaned by dipping it first in distilled water to remove as much proteinaceous material as possible so this will not coagulate at the orifice, then by dipping it in concentrated nitric acid, and then in water again. The mercury flow through the capillary should never be allowed to stop during this cleaning process.

To time the rate of the mercury drop formation, lower the capillary tube into the cell which has been filled with distilled water. Adjust the height of the mercury reservoir so that the arbitrarily chosen level is maintained. Without applying any voltage, observe the time required for 10 drops of mercury to fall. Repeat this, and check the time; so that observations agree within 0.1 second per drop. If the agreement is not this close, reclean the capillary tip with concentrated nitric acid. Cleaning of the capillary tip with nitric acid is usually necessary only at the beginning and end of a series of determinations.

*To leave the dropping mercury electrode overnight*, disconnect the galvanometer and attach the shunt provided. Leave the capillary standing in air. The mercury level in the reservoir tube can be lowered so that the rate of dropping will be reduced to zero, but we have found that the life of the capillary is prolonged if mercury is allowed to flow continuously. In any case, the mercury should never be allowed to recede from the capillary tip. Leave the electrolysis chamber full of distilled water; but adjust the mercury level so that it is just above the sintered glass disk. This protects the calomel electrode from diffusion of water through the agar.

*To Determine the Quantity of Oxygen in Solution the Procedure Is As Follows*—Connect the anode to the calomel half-cell and the cathode to the mercury in the capillary tube (the polarity should be reversed for certain electrooxidizable substances). Attach the resistance coil shunt to the galvanometer. Adjust the level of the mercury to a fixed mark in the standing reservoir above the capillary. Clean the capillary tip. Rinse the electrolytic cell well with distilled water. Determine the drop time.

Raise the leveling bulb so that the cell is filled with mercury; then insert the mercury-filled capillary into the cell. Recheck the mercury level in the stand-tube over the capillary. Make certain that no air bubbles are trapped in the cell and that there is a complete ring of mercury on top of the rubber tubing that holds the capillary tube in place. This is tested by applying a slight negative pressure. Draw the sample to be analyzed into a mercury-filled "blood sampling tube" and quickly connect this to the 3-way stop-cock in the cell. Open the stop-cock to the outside air and

expel a bit of the sample. Open the stop-cock to the cell and admit the sample. The sample should be drawn in until the mercury level is at the bottom of the reaction chamber (see 17, Fig. 2). If only an unusually small volume of fluid is available for analysis, the capillary can be lowered into the cell as far down as the mid-point of the sintered glass disk. It is important that the sample to be analyzed remain in contact with mercury as short a time as possible (see the section on the collection of material to be analyzed).

Close the stop-cocks, both in the cell and in the sampling tube, making certain at the time of closure that the leveling bulbs are adjusted to insure atmospheric pressure on the solution in the cell. Detach the sampling tube.

Set the potentiometer to apply 0.5 volt and read the galvanometer to tenths of a mm. scale division. The galvanometer oscillations which occur with each formation and fall of the mercury drop are averaged and the mean is taken as the correct value. If the swing is greater than 10 mm. or if the mid-point of the swing shifts, conditions are not satisfactory and must be checked. Break the contact and repeat the determination just made. The two values should check within 1 per cent.

To remove the sample from the cell, expel it through the capillary side arm by displacement with mercury. If the sample is needed for calibration, it is more saving of it to withdraw the dropping electrode from the cell and remove the sample with a pipette.

*To Determine Calibration Curve for Oxygen Dissolved in Lymph or Other Body Fluids*—When the entire current-voltage curve of a solution containing oxygen is obtained, as shown in Fig. 3, the current first increases rapidly then levels off for a time. It rises once more and then levels off again. The first plateau represents the reduction of oxygen to hydrogen peroxide and the second, the complete reduction to water.

The current registered by the galvanometer at any applied voltage on or between the two oxygen plateaus is proportional to the concentration of oxygen in the solution; therefore the practice of reading the current at only one voltage is followed. However, it is best to choose a point on one of the plateaus, since there the rate of increase in current per unit of applied E.M.F. is least; thus any error resulting from accidental voltage shifts is reduced to a minimum. In this set of experiments, the voltage chosen was that found at the mid-point of the oxygen to hydrogen peroxide plateau, 0.5 volt,  $E_{a.o.}$  versus saturated calomel electrode.

Since the current produced is known to vary directly with the concentration of dissolved oxygen, two points are adequate to establish the curve relating current to dissolved oxygen. (Several calibration curves were constructed by measuring a number of known oxygen concentrations. A

straight line relationship resulted in each case.) To establish the calibration curve, we have arbitrarily chosen zero concentration of oxygen and the oxygen concentration when the material being analyzed (lymph) was saturated with room air.

The room air sample was prepared by introducing about 2 cc. of lymph into a 20 cc. volumetric pipette. This resulted in a very shallow layer (a few mm. in depth) of lymph in the bulbous part of the pipette. Room air saturated with water vapor was then blown through the pipette over the solution. The pipette was gently rolled for 15 minutes at 25°,

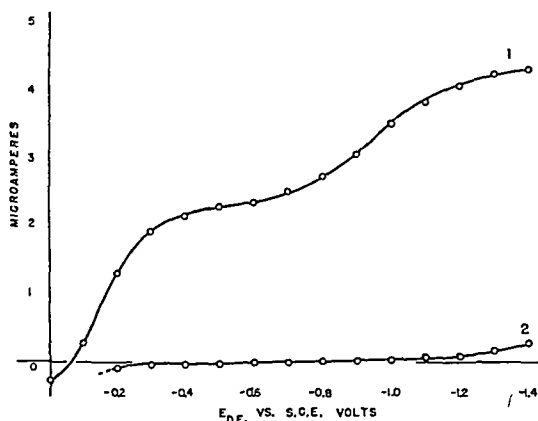


FIG. 3. Current-voltage curves of lymph showing typical "oxygen waves." The abscissa shows the applied voltage with the saturated calomel electrode (S.C.E.) as the anode and the dropping mercury capillary (d.e.) as the cathode. The ordinate represents microamperes. Curve 1 is for dog lymph saturated with room air. Curve 2 is the residual current of this lymph after the oxygen has been removed with sodium sulfite.

a fresh sample of air being blown through it from time to time. (10 minutes were established experimentally as adequate time for equilibration within the limits of error of the method.)

Zero concentration of oxygen was obtained by removal of the oxygen with sodium sulfite. This agent can be used only with solutions having a pH higher than 6.5. Negative catalysts which interfere with the sulfite reaction are present in some types of solutions. Their absence must be established. Sodium sulfite was added to a small amount of lymph to make a 1 per cent solution. The vessel was shaken, the surface of the lymph covered with oil, although this was probably not necessary with the sulfite present, and the sample set aside for 10 minutes to allow time for the re-

action to occur. This method of removing oxygen by sodium sulfite is simpler, quicker, and more complete than is equilibration with a stream of nitrogen (Kolthoff and Lingane (6) p. 310).

From these data, the calculation of the unknown oxygen concentration may proceed in two different ways. A calibration curve can be constructed by plotting the current found in the lymph containing sodium sulfite as zero oxygen concentration and the current in the lymph equilibrated with room air (20.94 per cent  $O_2$ ). From a straight line drawn between these two points, the solution containing an unknown oxygen concentration is read as a function of the current produced.

However, without construction of a calibration curve the unknown oxygen concentration can be calculated by employing the Ilkovic equation,  $i_d = kC$ ,<sup>3</sup> in which  $i_d$  is the diffusion current corrected for the residual current and  $C$  is the observed galvanometer reading less the current in the oxygen-free (treated with  $Na_2SO_3$ ) solution;  $k$  for the capillary (drop time and pressure remaining constant) is established by relating  $i_d/C$  for the sample equilibrated with room air. By means of this value of  $k$ ,  $C_1$ , the concentration of the unknown, is determined.

*To Collect Material to Be Analyzed*—The method of collecting and storing the samples of body fluid to be analyzed for oxygen was studied with considerable care. Collection over mercury was found to be unsatisfactory. Lymph showed a steadily decreasing oxygen content when allowed to stand over mercury. This is possibly due to the reaction of mercury with the chloride ions to form mercurous chloride, oxygen being reduced in the process (see Furman and Murray (3)). In the case of lymph, catalysts may possibly be present to speed up the reaction.

Collection of the lymph under mineral oil also has hazards, as there is a diffusion gradient across the oil tending to establish equilibrium between the sample and the outside air. The rate of diffusion, however, is slow. We have found that the resultant error could be reduced to a negligible minimum by taking precautions as follows: The samples to be analyzed were collected under mineral oil and the approximate oxygen concentration determined. Oil was then equilibrated with oxygen at this tension. This oil was then used to protect the samples used for the accurate determination of the oxygen concentration.

The material to be analyzed was collected in long (12 cm.), narrow (8 mm.) bore tubes. At the beginning of collection such a tube was filled with the oil which had been equilibrated with oxygen at the approximate tension of the sample to be analyzed. The sample was added at the bottom. The top oil which had come into contact with the room air was displaced as the sample was added. An oil barrier at least 2 or 3 cm. high

<sup>3</sup> Kolthoff and Lingane ((6) p. 59).

was maintained between the sample and the room air. The sample was withdrawn from the bottom of the tube and the top several mm. which had been in contact with the oil were discarded.

The sample was iced to diminish the oxygen consumption of the few cells present in the case of lymph. These were centrifuged off as soon as possible, since the collection of lymph is rather slow. The oxygen concentration was determined immediately after an adequate sample had been collected.

TABLE I

*Oxygen in Gas Mixture Determined with Haldane Apparatus and Dropping Mercury Electrode*

Haldane apparatus	Dropping mercury electrode	Haldane apparatus	Dropping mercury electrode
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
29.8	28.5	11.9	13.0
20.9	21.5	10.1	9.5
20.9	21.1	9.0	10.0
20.9	21.0	9.0	7.0
20.9	20.7	6.4	6.8
15.1	15.4	4.8	4.0
14.4	14.0	4.5	3.9
12.2	12.7	3.5	5.6

#### *Accuracy of Method*

To check the accuracy of this method the following procedure was carried out. Lymph from dogs was equilibrated at 25° with various oxygen-nitrogen mixtures. 5 cc. of lymph were rotated in a 50 cc. tonometer. A given gas mixture was saturated with water vapor at 25° and then passed through the tonometer. Under these circumstances equilibration within the experimental limits of the analytical method occurred in 10 minutes; however, each equilibration procedure was carried out for 15 minutes. The following consecutive samples were examined in random order. The operator of the dropping mercury electrode did not know the oxygen content of the gas mixture that had been equilibrated with the lymph being examined. The Haldane determination is of course direct and the dropping mercury electrode indirect. Room air oxygen was taken as the standard for calibration. The oxygen percentage of the gas mixture in equilibrium with the lymph was found in the various cases to be as shown in Table I.

#### SUMMARY

Polarographic analysis has been adapted to the determination of low tensions of oxygen in small quantities of body fluids.



We wish to acknowledge our indebtedness to Dr. James J. Lingane for many helpful suggestions during the course of this work.

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# THE NON-UTILIZATION OF LACTIC ACID BY THE LACTATING MAMMARY GLAND

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Following the reports of Graham (3) and Shaw, Boyd, and Petersen (6) it has been generally accepted that the lactating mammary gland utilizes lactic acid. These reports were based on arteriovenous differences in which it was found that the mammary venous blood usually contained less lactic acid than the arterial blood.

The arteriovenous lactic acid differences reported are extremely variable, especially for the goat. Graham's data on goats show the relatively high average utilization of 16.7 mg. per cent with a range of 0.1 to 57.9. The arterial values appear unusually high, averaging 58.0 mg. per cent with a range of 10.0 to 134.3. The data of Shaw *et al.* on cows were also variable, showing a mean utilization of 3.64 mg. per cent with values ranging from a negative utilization of 1.55 to a positive utilization of 10.42 mg. per cent. The arterial values varied from 3.93 to 19.46 mg. per cent.

It appeared advisable to attempt to obtain more nearly constant lactic acid differences which might be expected to be more representative of the normal metabolism of the gland. It was suspected that the large variations previously observed were due primarily to excitation of the animal during the drawing of the blood samples. Accordingly a reexamination was made of the rôle of blood lactic acid in milk secretion in which the usual method (Shaw and Petersen (7)) was used to determine whether the animal had been unduly disturbed during the drawing of the blood samples.

## EXPERIMENTAL

The arterial and mammary venous blood samples were drawn as in previous experiments, the samples being collected in oxalated vessels containing ammonium fluoride and chilled in ice water. Lactic acid determinations were conducted immediately after the samples were drawn. The method of Miller and Muntz (5) as modified by Koenemann (4) and Barker and Summerson (1) was used for lactic acid. Hemoglobin was determined by the method of Evelyn and Malloy (2).

\* The experimental data in this paper are taken in part from a thesis presented by Ross C. Powell, Jr., in partial fulfilment of the requirements for the degree of Master of Science in Dairy Industry, University of Connecticut, Storrs Agricultural Experiment Station.

The results of thirty-four experiments on cows are shown in Table I. All animals showing changes in mammary blood concentration in excess of 0.5 per cent were considered as unduly excited. In seventeen experiments, falling in this category, there was an apparent mean utilization of 2.40 mg. per cent of lactic acid. The standard error of 0.70 demonstrates that this difference is highly significant. However, in seventeen experiments in which the blood concentration changes were 0.5 per cent or less,

TABLE I

*Arteriovenous Lactic Acid Differences in Lactating Glands of Cows As Related to Arteriovenous Hemoglobin Concentration*

The values represent blood lactic acid measured in mg. per cent.

Changes in Hb concentration, 0.5 per cent or less				Changes in Hb concentration in excess of 0.5 per cent			
Cow No.	Arterial	Venous	Difference	Cow No.	Arterial	Venous	Difference
1	9.1	7.9	1.2	4	11.0	6.0	5.0
2	6.7	6.3	0.4	7	5.4	6.8	-1.4
3	5.9	5.6	0.3	8	5.9	4.1	1.8
5	4.8	4.9	-0.1	16	14.6	5.2	9.4
9	6.8	6.7	0.1	17	8.6	5.3	3.3
10	5.6	5.4	0.2	18	8.0	5.8	2.2
C-1	11.1	11.0	0.1	19	7.4	4.0	3.4
C-2	10.4	9.0	1.4	20	8.3	6.3	2.0
C-3	10.0	13.2	-3.2	C-2	11.4	10.1	1.3
S-2	11.1	9.0	2.1	C-2	11.4	10.7	0.7
S-3	7.1	6.6	0.5	C-2	12.4	13.3	-0.9
K-2	4.9	4.4	0.5	C-4	10.0	8.6	1.4
K-1	2.6	3.2	-0.6	K-5	14.6	9.8	4.8
K-3	6.4	6.4	0.0	K-6	10.6	6.9	3.7
K-4	8.6	6.6	2.0	S-1	10.6	13.0	-2.4
K-2	7.2	4.6	2.6	S-1	11.0	6.0	5.0
K-6	5.6	4.2	1.4	S-4	10.0	8.3	1.7
Average.	7.29	6.77	0.52 ± 0.32		10.07	7.66	2.40 ± 0.70

indicating little or no excitation, the mean utilization of lactic acid was only 0.52 mg. per cent. The standard error of 0.32 shows that this difference is not statistically significant. Therefore, it appears that the reported utilization of lactic acid is only an apparent utilization due to excitation. The mean arterial concentration of lactic acid in the group showing evidences of excitation was 10.07 mg. per cent, whereas that of the undisturbed group was only 7.29 mg. per cent.

That the active mammary gland does not normally use blood lactic acid was further confirmed by experiments on animals under nembutal anesthe-

sia. Three cows and a goat were anesthetized by injecting nembutal intravenously.

From 8 to 10 gm. of nembutal were required to keep a cow weighing approximately 1200 pounds completely anesthetized for a period of 45 minutes. Usually an initial intravenous injection of from 4 to 5 gm. was given, after which an additional gm. was injected every 15 minutes. The goat was anesthetized with 2 gm. of nembutal injected intravenously. An additional gm. was injected intraperitoneally to keep the animal anesthetized for the required period. Two consecutive arteriovenous lactic acid differences were determined on each animal at the time intervals indicated in Table II. In each of the four experiments there was an apparent utilization of lactic acid when the animals had been anesthetized

TABLE II  
*Arteriovenous Lactic Acid Difference in Lactating Glands of  
Animals under Anesthesia*

Animal No.	Time under anesthesia	Difference in arteriovenous Hb concentration	Blood lactic acid		
			Arterial	Venous	Difference
	<i>min.</i>	<i>per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
Cow 1	15	0.00	13.0	8.3	4.7
	18	0.00	10.0	8.3	1.7
" 2	13	0.00	11.2	7.7	3.5
	55	0.00	7.4	7.7	-0.3
" 3	13	2.03	19.4	11.7	7.7
	38	0.00	8.0	8.3	-0.3
Goat 1	20	0.00	7.8	5.2	2.6
	30	0.00	5.3	4.8	0.5

for 20 minutes or less. However, the arteriovenous difference was not significant when the samples were taken after the animals had been anesthetized for periods of 30 to 55 minutes. In each case there was a substantial decrease in the lactic acid in the arterial blood as the period of anesthesia increased. These data support the suggestion that the utilization of lactic acid reported previously was not a true utilization but rather an apparent utilization associated with excitation.

#### DISCUSSION

It is believed that the apparent utilization of lactic acid with excitation is due to a sudden increase of lactic acid in the blood in which there is a rapid diffusion of lactic acid into the glandular tissue, resulting in a temporary disproportion in the lactic acid concentration of the blood traversing the mammary gland.

It is apparent that changes in the concentration of lactic acid in the blood traversing the gland provide an even more sensitive indication of disturbance to the animal than changes in hemoglobin concentration. The data in Table I show that the arterial level of lactic acid was occasionally higher than the venous level even though the hemoglobin concentration change was not in excess of 0.5 per cent. This probably indicates a mild degree of excitation resulting in a slight increase in the lactic acid of the blood which was not reflected in the mammary venous blood because of the rapid diffusion of lactic acid into the glandular tissue. The disturbance was not sufficient, however, to affect the concentration of the blood passing through the gland. The lactic acid of the arterial blood flowing into the gland is so markedly affected by excitation that the disproportion between the arterial and venous blood is undoubtedly due primarily to the sudden change in the concentration of lactic acid in the arterial blood. As considerable time must elapse before the blood lactic acid returns to normal, it is not surprising that lactic acid continues to pass into the mammary tissue for some time following the initial excitation of the animal.

#### SUMMARY

Blood lactic acid is not normally utilized by the lactating mammary gland. The utilization previously reported was apparently due to excitation of the experimental animals and is not believed to represent a true utilization.

The average lactic acid content of arterial blood samples drawn from seventeen cows, without any appreciable disturbance to the animals, was 7.29 mg. per cent.

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## LIPID OXIDASE STUDIES

### II. THE SPECIFICITY OF THE ENZYME LIPOXIDASE\*

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(Received for publication, August 29, 1942)

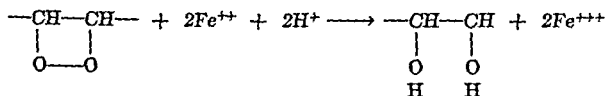
It was observed by Sumner and Sumner (1) that the so called "carotene oxidase," found in soy beans, oxidized carotene only in the presence of unsaturated fats. This observation was later confirmed by the independent investigations of Tauber (2). The oxidizing factor was identified as the enzyme lipoxidase, the existence of which was first noted by André and Hou (3).

The object of this work was to determine the effect of this enzyme on various unsaturated compounds, in order to establish the structure or structures for which the enzyme is specific. The unsaturated compounds used were those available commercially or those easily prepared from natural sources.

#### EXPERIMENTAL

The unsaturated compound under investigation was dissolved in alcohol or acetone, this solution was diluted with 20 parts of water, and the resulting colloidal suspension was treated with an aqueous extract of fat-free soy bean meal.

The effect of the enzyme extract on the unsaturated compounds was measured by means of a colorimetric determination of the amount of peroxidation produced (4). These organic peroxides proved capable of oxidizing ferrous iron, and the ferric iron formed was determined as the thiocyanate with the aid of a photoelectric colorimeter. The maximum peroxidation obtained in each case was calculated as the percentage of theoretical total peroxidation for the compound. The calculations were based on the following relationship (5).



Allyl alcohol, an unsaturated alcohol possessing one double bond and 3 carbon atoms, was not peroxidized by lipoxidase. Nor was any peroxidation observed when the determinations were repeated with mesityl oxide,

\*Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 509, May 9, 1942.

a 6-carbon unsaturated ketone, or with linalool, an unsaturated tertiary alcohol, 3,7-dimethyloctadiene-1,6-ol-3.

Since the enzyme showed no activity toward this heterogeneous group of structures, several fatty acids were tried.

Oleic acid was peroxidized by the soy bean extract, maximum peroxidation having been obtained in 5 minutes. The value for the maximum was found to be 16 per cent of the theoretical. Elaidic acid, the *trans* isomer of oleic acid prepared from it by the action of nitrous acid, was not peroxidized.

Erucic acid, the *cis* form of the unsaturated acid having a double bond in the 13-14 position, was not affected by the soy bean extract.

Linoleic acid was peroxidized with great rapidity, the maximum value being over 90 per cent of the theoretical total peroxidation of one double bond, or over 45 per cent of the theoretical total for both double bonds. Linoleic acid is unsaturated at carbon atoms 9 and 12, and is the higher melting form, indicating a *cis* configuration.

The oleic, erucic, and linoleic acids were purchased from a chemical supply house.

$\alpha$ -Eleostearic acid, the *cis* isomer possessing conjugated unsaturation in the 9, 11, and 13 positions, proved to be susceptible to enzymic peroxidation. The activity was of the same order as that shown by oleic acid. The total peroxidation was the same as that obtained with oleic acid, the maximum value being about 16 per cent of theoretical for one double bond, but only about 5 per cent of the possible peroxidation on the basis of three double bonds. The maximum was attained in 10 minutes. This compound was prepared from China wood oil, according to the method of Thomas and Thomson (5).

Linolenic acid, triply unsaturated in the 9, 12, and 15 positions, was peroxidized very rapidly, the effect being similar to the results obtained with linoleic acid. Less time was required to reach maximum peroxidation, but the extent of the reaction was about the same, the maximum value being 95 per cent of the theoretical value for the total peroxidation of one double bond. The linolenic acid was prepared from a mixture of the fatty acids distilled from linseed oil. The ether-insoluble hexabromide was isolated and treated with zinc dust and acidified methyl alcohol. The methyl ester was saponified, the soaps were acidified, and the organic acid was extracted with ether. Evaporation under a vacuum yielded linolenic acid as a pale yellow liquid. The iodine number (Hanus) was 261.

The action of the enzyme on glycerides of the unsaturated fatty acids was demonstrated by using technical triolein and an unrefined preparation of the acid-free glycerides of linseed oil. These glycerides were peroxidized at the same rate and to the same extent as were the corresponding crude fats.

## DISCUSSION

The reaction with oleic acid shows that the enzyme will peroxidize a double bond of the *cis* configuration in the 9-10 position, while the fact that no peroxidation is produced with erucic acid suggests a specificity for the 9-10 position. The absence of activity toward elaidic acid indicated that lipoxidase is stereospecific, requiring the *cis* configuration of the double bond.

The presence of an additional isolated double bond greatly increases both the rate and the extent of the peroxidation, as is shown by the work with linoleic acid. However, a compound containing three isolated double bonds, linolenic acid, was peroxidized to the same degree, and at a slightly increased rate. This indicates that the effect of added isolated double bonds in a fatty acid showing unsaturation in the 9-10 position is merely to increase the reactivity of that position, since an increase in peroxidation would be expected in the case of the linolenic acid were more than one double bond attacked. That this acceleration is not produced by the presence of conjugated unsaturation is shown by the diminished effect on  $\alpha$ -eleostearic acid.

## SUMMARY

The relative effect of lipoxidase on various unsaturated compounds was investigated. The results of this work indicate that the enzyme is most effective on the structural group



the double bond nearer the carboxyl group being probably of the *cis* configuration.

These results agree in part with the observations of Spoehr, Smith, Strain, and Milner (6, 7). These workers, in a preliminary report of independent investigations on this problem, found the fat-peroxidizing enzyme to be effective on compounds similar in structure to oleic acid. They did not report the relative specificity described in this work. However, their method of operation, in which the measurement of oxygen absorption by unsaturated compounds was employed, involved a system vastly different from the aqueous suspensions used in this study.

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## LIPID OXIDASE STUDIES

### III. THE RELATION BETWEEN CAROTENE OXIDATION AND THE ENZYMIC PEROXIDATION OF UNSATURATED FATS\*

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(Received for publication, August 29, 1942)

Sumner and Sumner (1) demonstrated that the oxidation of carotene by an enzyme from the soy and other beans is dependent upon the presence of unsaturated fats. These unsaturated fats are converted into peroxides by the enzyme lipoxidase (2) and the carotene is oxidized simultaneously. Later Tauber (3, 4) suggested that the carotene oxidation was brought about by the action of fat peroxides on carotene. At first sight Tauber's proposal appears credible. However, evidence offered in this paper and in the earlier work (1) proves that the mechanism is quite different.

#### EXPERIMENTAL

Linseed oil was highly peroxidized by stirring violently with aqueous soy bean meal extracts and the peroxidation of the product was then determined by centrifuging off the oil, adding glacial acetic acid, chloroform, and saturated aqueous potassium iodide, and titrating the free iodine with thiosulfate. When carotene suspensions were stirred with this peroxidized fat, there was a decolorization of the carotene only after several hours. Various media were employed, including aqueous suspensions and heavy fat emulsions.

Since peroxidized fats have little immediate action on carotene, it remained to be shown that there exists in soy bean meal no enzyme which catalyzes the oxidation of carotene by fat peroxide. It was observed that highly active preparations of horseradish peroxidase could indeed catalyze the oxidation of carotene by peroxidized fat, but that this reaction proceeded very slowly. The following experiment attests that soy meal contains no other enzyme capable of transferring oxygen from peroxidized fat to carotene.

*Solution A*—To 5 mg. of fresh linseed oil in 5 cc. of acetone were added 100 cc. of water, 5 cc. of phosphate buffer of pH 6.5, and 2 cc. of soy meal extract (1 gm. of meal in 50 cc. of water). The suspension is then allowed to stand for 5 minutes to permit the fat to become peroxidized.

\* Approved by the Director of the New York State Agricultural Experiment Station as Journal Paper No. 510, May 9, 1942.

*Solution B*—This is the same as *Solution A*, but without any soy meal extract.

After the 5 minute period 2 cc. of soy meal extract were added to *Solution B* and 5 cc. of carotene in acetone were added to both suspensions. In *Solution B* the carotene was completely decolorized in 4 minutes, while in *Solution A* the carotene was decolorized in 35 minutes.

From this experiment it is obvious that the rapid oxidation of carotene is brought about neither by the direct action of fat peroxides nor by the intervention of an enzyme which carries oxygen from the fat peroxides to carotene. It appears instead that oxidation of unsaturated fat must be

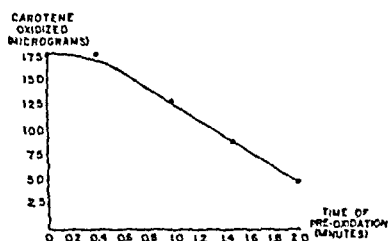


FIG. 1

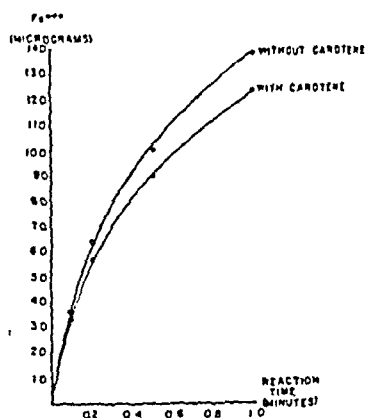


FIG. 2

FIG. 1. Effect of preoxidation of unsaturated fatty acid on carotene oxidation.

FIG. 2. Effect of the presence of carotene on the enzymic oxidation of unsaturated fat.

occurring if the rapid oxidation of carotene is to be produced by this system. A second experiment was as follows:

With 5 mg. aliquots of linoleic acid as the unsaturated factor, a series of determinations was run in which peroxidation was allowed to proceed for various periods of from 0.4 to 2.0 minutes before the carotene was added. 2 minutes after the carotene was added, the reaction was stopped by the addition of concentrated hydrochloric acid and the amount of carotene remaining was determined colorimetrically. Fig. 1 shows the curve obtained by plotting the quantity of carotene oxidized against the time of preoxidation. This shows graphically that fat oxidation must be actually occurring in order to produce carotene oxidation. When the preoxidation has progressed to such an extent that the fat or fatty acid has been largely peroxidized, carotene is not oxidized.

The author has developed a method (5) for following the peroxidation

of small quantities of fat in aqueous suspension. The method is capable of showing clearly the linear dependence of carotene oxidation upon the oxidation of unsaturated fat. Here, lipoxidases from several sources were tested with a variety of unsaturated organic compounds. It was found that conditions which slowed the rate of peroxidation caused a greater carotene oxidation per mg. of peroxide formed. This agrees with the "Skrabal rule" for induced reactions (6).

Further work showed that when carotene was added the amount of peroxidation of the unsaturated fat was not so great as in the case when no carotene was added. As Fig. 2 illustrates, this phenomenon is quantitative. Here, the extent of peroxidation is expressed in micrograms of ferric iron formed in the analyses.

It has been possible to show that inorganic oxidizing agents can produce the same effect upon carotene as lipoxidase. The following test was run.

1. To a suspension of unsaturated fat and carotene a few drops of fuming nitric acid were added. The carotene was completely bleached in 90 seconds.

2. A few drops of fuming nitric acid were added to a suspension of carotene in water. The carotene was completely oxidized in 45 minutes. A similar result was obtained with permanganate, but here it was necessary to extract the carotene, since the color of the permanganate obscured the carotene color.

#### DISCUSSION

Since carotene is not directly oxidized by this enzyme and since the fat peroxides do not cause the oxidation, there arises the possibility that the oxidation of the carotene is brought about by an oxidizing agent formed as an unstable intermediate during the production of the fat peroxide. This hypothetical reactive intermediate oxide has been suggested as a possible mechanism for certain induced reactions (6) and, according to Kolthoff and Menzel (7), it was originally suggested by W. Manchot that in all oxidation processes primary oxides are formed and that these are often stronger and quicker oxidizing agents than the substances from which they are formed.

#### SUMMARY

Evidence has been submitted that carotene is not oxidized by the peroxides formed from unsaturated fat either directly or indirectly.

It has been shown that the oxidation of carotene requires that the peroxidation of unsaturated fat be actually in progress.

Since fat peroxidation is diminished by the coupled oxidation of carotene, it appears possible that the oxygen which reacts with carotene is derived from some intermediate which is produced during the peroxidation of the unsaturated fat.

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## BIOCHEMISTRY OF FUSARIA

### THE INFLUENCE OF DIPHOSPHOPYRIDINE NUCLEOTIDE ON ALCOHOLIC FERMENTATION (IN VIVO)

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(Received for publication, August 31, 1942)

The changes in phosphorus distribution during fermentation in the living yeast cell appear to be consistent (1) with the theory of alcoholic fermentation developed from experiments on cell-free extracts (2) and there is strong suggestion that the processes are analogous. There are, however, a number of differences between fermentation by the living cell and cell-free preparations. Among these is the fact that the rate of fermentation by living cells is uninfluenced by added diphosphopyridine nucleotide (coenzyme I). This may be a reflection of the organized nature of cells containing enzyme systems already saturated with respect to coenzyme I and protected by cell membranes. As a result there is but scanty evidence to provide a basis for the assumption that the fermentation of sugar by living cells takes place as in cell-free extracts through the formation and decomposition of phosphoric esters, phosphorus transference, and oxidation-reduction reactions.

The genus *Fusarium* (Link) is characterized biochemically (3) by the very slow but almost perfect manner in which these organisms ferment hexoses and pentoses to ethyl alcohol and  $\text{CO}_2$ . The slow rate of *Fusarium* fermentation may be due to a marked deficiency in certain components of the fermenting system; a study of this slow fermentation may indicate the rôle (*in vivo*) of such components in both yeast and *Fusarium* fermentation in view of their striking similarity (4).

Studies of inhibition of *Fusarium* fermentation by fluoride (4) have yielded results almost identical with those observed with living yeast (1). The ultimate effect of NaF on cell-free fermentation is that the fermentation cycle is immobilized as soon as all of the diphosphopyridine nucleotide (DPN) has been reduced by triose phosphate, with the formation of phosphoglyceric acid. This suggests strongly that oxidation and reduction of DPN may play a significant and perhaps limiting rôle in *Fusarium* fermentation. Dietrich and Klammerth (5) have studied the effects of vitamin B preparations and of nicotinamide on pentose fermentation by *Fusarium lini*. They have reported stimulating effects by nicotinamide which strongly suggest that its action may be the result of its availability for pyridine nucleotide synthesis.

In the present investigation, the rôle *in vivo* of diphosphopyridine nucleotide (coenzyme I) in alcoholic fermentation by *Fusarium tricothecoides* has been studied.

#### EXPERIMENTAL

*Organism and Culture Medium*—The organism used in the experiments is catalogued in the Massachusetts Institute of Technology culture collection as *Fusarium* sp. H. A study of its morphological characteristics indicates that it is closely allied to *Fusarium tricothecoides*. It was selected because of the uniformity of its growth and biochemical characteristics.

The basal salt solution used for the growth medium was made up of  $\text{NaNO}_3$  2,  $\text{KH}_2\text{PO}_4$  1,  $\text{KCl}$  0.5, and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 gm. per liter. The required amounts of glucose, dried yeast extract (Disco), and diphosphopyridine nucleotide or nicotinic acid were added in sterile condition when necessary. Cultures were grown in 1 liter conical flasks each containing 300 cc. of the medium. Sterilization was effected by autoclaving at 15 pounds pressure for 20 minutes. The flasks were inoculated with 1 cc. of a suspension of spores and the mycelium prepared by scraping into 100 cc. of sterile water the growths from two or three glucose-basal salt agar slants grown in test-tubes for 5 to 6 days. The cultures were incubated at  $24^\circ$ .

*Qualitative Identification of Diphosphopyridine Nucleotide in Fusarium tricothecoides*—A number (80) of flasks of 3 per cent glucose-basal salt medium were inoculated with a spore-mycelium suspension of the organism and incubated for 7 to 10 days. The growths were removed from the medium, pressed lightly, washed, and then extracted with an equal weight of water or  $0.1 \text{ M } \text{KH}_2\text{PO}_4$  at  $80^\circ$  for 20 minutes. Positive qualitative evidence of the presence of pyridine nucleotides was obtained by replacing the coenzyme with aqueous extracts of *Fusaria* in a number of test systems. Positive tests were obtained by the apozymase method of von Euler and Myrbäck (6) with coenzyme-free, washed, dried yeast, by the lactic dehydrogenase method of Green and Brosteaux (7) with washed heart muscle, by the method of Lwoff and Lwoff (8) by determining the stimulating effect on growth of *Hemophilus parainfluenzae*, and by the chemical detection of nicotinic acid (9). The results in every instance indicated a relatively low concentration of pyridine nucleotides in *Fusarium tricothecoides* grown under the conditions described.

*Influence of Diphosphopyridine Nucleotide on Alcohol Production*—The small amount of diphosphopyridine nucleotide observed in the qualitative tests and also in the quantitative estimations (Table I) suggested that normally there may be a deficiency of this component in *Fusaria* which might account for the slow fermentation that characterizes these organisms. To test this possibility a number of flasks each containing 300 cc. of the

basal salt to which were added 3 per cent glucose and a desired amount of diphosphopyridine nucleotide,<sup>1</sup> nicotinic acid, or yeast extract (Difco) were inoculated with *Fusarium tricothecoides*. Periodically, small samples of culture fluid were withdrawn and ethyl alcohol was estimated by the method of Friedemann and Klaas (10). Typical results of such experiments are indicated in Fig. 1 and show clearly that diphosphopyridine nucleotide in rather small amounts appreciably stimulates fermentation. Stimulation is also apparent when the medium is supplemented with

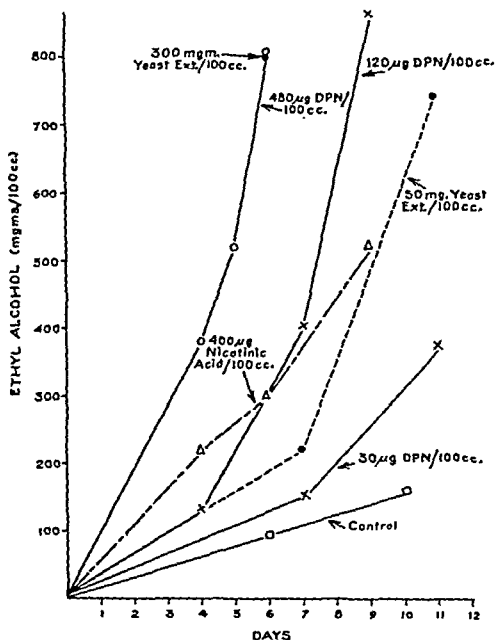


FIG. 1. The influence of yeast extract, nicotinic acid, and diphosphopyridine nucleotide on ethyl alcohol production *in vivo* by *Fusarium tricothecoides*.

nicotinic acid but not to the same degree as with the DPN, which suggests that the action of nicotinic acid may be due to its availability for pyridine nucleotide synthesis. It is not unlikely that the growth-stimulating effects of nicotinic acid on a number of bacteria (11-13) may be due to a similar effect. In all of the present experiments no stimulation of growth was observed by either DPN or nicotinic acid. However, if the source of

<sup>1</sup> Supplied through the courtesy of Dr. B. Jandorf, who also carried out the DPN estimations given in Table I, and to whom we express our thanks.



coenzyme was dried yeast extract, both growth and alcohol production were stimulated; this might be expected in view of the numerous growth factors contained in yeast extract. Once growth is established, the rate of alcohol production of the coenzyme-supplemented medium increases to as much as 20 times the control rate (Fig. 1).

*Estimation of Diphosphopyridine Nucleotide in Fusarium tricothecoides*—The oxidized diphosphopyridine nucleotide content of the organisms grown on normal and yeast extract media was estimated by the method of Jandorf *et al.* (14). The values obtained are given in Table I. It is apparent from these figures that increased alcohol production is associated with increased coenzyme content. Culture G-2 (Table I) is extremely interesting in this respect. This culture was grown on the unsupplemented glucose medium and during the first 10 days of growth it followed the normal control curve characterized by low alcohol yield and low coenzyme

TABLE I

*Relation of Alcohol Production by Fusarium tricothecoides to Diphosphopyridine Nucleotide Content*

Culture No.*	Age	Ethyl alcohol	DPN	Weight of mat per flask	DPN per culture
	days	mg. per 100 cc.	$\gamma$ per gm.	mg.	$\gamma$
G-1	7	100			
G-2	10	150	30.7	220	6.75
G-2	14	750	95.0	209	20.0
Y-3	3	815			
Y-0.3	10	†	167.0	1500	251

\* G = glucose culture, Y = glucose-yeast extract culture.

† Alcohol largely consumed after the 3rd day.

content. From the 10th to 14th day, however, the rate of alcohol production increased markedly and an estimation of the coenzyme content at this time indicated a concomitant rise in diphosphopyridine nucleotide. Growths on the yeast extract medium had the highest coenzyme content, as would be expected from the high content of coenzyme and nicotinic acid in the yeast extract.

In view of the high DPN content of the growths on yeast extract media a series of flasks of 3 per cent glucose-0.3 per cent yeast extract medium was inoculated and incubated for 6 to 7 days. The medium was then drawn off and the growths carefully washed under aseptic conditions until no sugar or alcohol could be detected in the washings. 300 cc. quantities of sterile medium containing either 3 or 10 per cent glucose or 3 per cent sucrose were introduced under the washed growths. Alcohol was estimated periodically and the results are shown in Fig. 2. It is apparent that

the yeast growths ferment either glucose or sucrose at a rate comparable to the rate of fermentation of coenzyme-supplemented growths once they have been established (Fig. 1). The rate of fermentation is about 20 to 25 times that of the controls. Repeated experiments under these conditions gave results of unusual uniformity in respect to alcohol production. The rate for *Fusarium tricothecoides* was generally about 300 mg. of ethyl alcohol per day per 100 cc. of medium if adequate substrate was present. In the absence of the substrate the alcohol was rapidly dissimilated. The

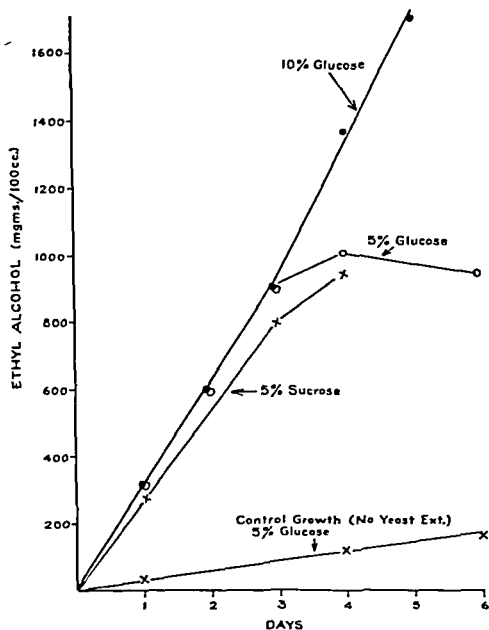
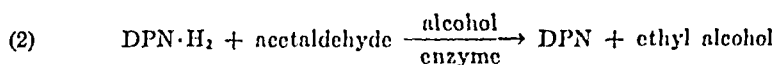
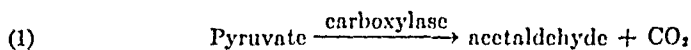


FIG. 2. The effect of dried yeast extract on the fermentation of glucose and sucrose by *Fusarium tricothecoides*.

fact that the DPN-stimulated cultures showing no increase in growth gave alcohol yields equal to those of the yeast extract cultures indicates that the components in yeast extract which stimulate growth apparently have little effect on alcohol production, except in so far as they make possible the assimilation of the DPN and nicotinic acid of the yeast extract by the organism. The increase in alcohol production appears to be related directly to the increase in DPN.

In contrast to the observations made recently on *Fusarium lini* Bolley,

by Wirth and Nord (15), the present investigators while demonstrating the presence of a carboxylase system in *Fusarium tricothecoides* could not demonstrate (16) any stimulating effects of thiamine, or increased co-carboxylase content of *Fusarium tricothecoides*, on alcohol production. This may be due to inherent differences in the metabolism of these organisms. In spite of these differences, it appears highly probable that in *Fusarium* fermentation ethyl alcohol arises *in vivo* according to the reactions



#### SUMMARY

The rôle *in vivo* of diphosphopyridine nucleotide (coenzyme I) has been studied and it has been shown to be a limiting factor in alcohol production by *Fusarium tricothecoides*. This suggests that fermentation by living cells is similar to that observed in cell-free preparations involving oxidation-reduction reactions and phosphorus transfer.

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# UROCANIC ACID AND THE INTERMEDIARY METABOLISM OF HISTIDINE IN THE RABBIT\*

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(Received for publication, September 17, 1942)

Urocanic acid, discovered in 1874 by Jaffe in dog urine (1), has been an evasive compound. The dog from whose urine it was first isolated escaped, and Jaffe was unable to detect urocanic acid in the urine of other dogs or of humans. Subsequently it was observed in the urine of two other dogs (2), but was not found to be a regular constituent of canine or human urine (2, 3). The isolation of an acid similar to Jaffe's urocanic acid from the urine of one species of coyote (4) was not confirmed with the urine of a different species of coyote (5). After the chemical structure of urocanic acid as  $\beta$ -[4(5)-imidazolyl]acrylic acid, the  $\alpha, \beta$ -unsaturated acid corresponding to histidine, had been established (3), Raistrick obtained the acid by the action of bacteria of the *coli-typhosus* group on histidine (6). The suggestion has been made that the animals which excreted urocanic acid presented a rare anomaly of metabolism (3, 7), possibly related to its formation from histidine by microorganisms in the large intestine.

Urocanic acid has also been obtained by Hunter from a pancreatic digest of casein (3), but here also the compound was elusive, since in repeated attempts he was unable to obtain the compound from digests again. The experiments of Raistrick have not been confirmed, and Raistrick himself was not able to extend his findings to other bacteria (8).

Kotake and Konishi (9), Konishi (10), and Kiyokawa (11) have suggested that urocanic acid is a product of the normal metabolism of histidine in dogs and rabbits, and were able to isolate the acid from the urine after oral or parenteral administration of histidine. Harrow and Sherwin (12) considered that urocanic acid was a possible intermediate in the normal catabolism of histidine. Cox and Rose (13) were unable to demonstrate that urocanic acid was effective in the promotion of growth of young white rats fed a diet deficient in its content of histidine, while Harrow and Sherwin observed "slight beneficial effects" of urocanic acid on growth under these dietary conditions.

\* A part of the work here reported was carried out in the Laboratories of the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, during the tenure of a National Research Council Fellowship in the Medical Sciences awarded to one of the authors (W. J. D.).

The experiments to be described are concerned primarily with the excretion of urocanic acid by the rabbit after the administration of histidine and the fate of the acid when fed or injected into the same species. It is believed that they afford little evidence that urocanic acid is an important intermediary product of the metabolism of histidine.

#### EXPERIMENTAL

*Preparation of Histidine*<sup>1</sup>—The *l*-histidine monohydrochloride was isolated from blood paste by the procedure of Foster and Shemin (14). Total and amino (Van Slyke) nitrogen determinations on two lots were 19.8 and 20.1, and 6.8 and 6.8 per cent respectively (theoretical, 20.04 and 6.68 per cent). Both samples gave definitely positive cyanide-nitroprusside tests for the disulfide linkage. The specific rotations determined in the presence of 3 moles of hydrochloric acid at 26° were +7.0° and +7.2°. A sample which had been recrystallized six times until the cyanide-nitroprusside test was negative showed an  $[\alpha]_D^{20}$  of +8.0°, in agreement with the chief reported values (14, 15) for these experimental conditions.

We have tested five samples of histidine monohydrochloride prepared in this laboratory, two commercial samples of this salt, and one commercial sample of histidine dihydrochloride for the presence of cystine. All the samples gave negative ammonia-nitroprusside, positive cyanide-nitroprusside, and positive Sullivan naphthoquinone tests. The intensity of the latter reaction paralleled that of the cyanide-nitroprusside test. Tests for alkali-labile sulfur and for sulfur after fusion with sodium were positive. No sulfide test could be obtained prior to sodium fusion. It appears therefore that cystine may be present in small amounts as an impurity in preparations of histidine. The intensity of the cyanide-nitroprusside test given by the various samples corresponded roughly to those given by pure cystine solutions to indicate a content of 0.5 to 2.0 per cent of "cystine" in the histidine monohydrochloride. This estimate is in accord with the slightly low values for specific rotation obtained, values which might be expected if cystine with its levorotation were present in small amounts. It was possible by repeated recrystallization to obtain samples which showed no reaction with cyanide and nitroprusside. More efficient purification was obtained by the conversion of the monohydrochloride to the dihydrochloride.

*Preparation of Urocanic Acid*<sup>1</sup>—Urocanic acid was synthesized by the method of Barger and Ewins as given in detail by Cox and Rose (13) and also by the decarboxylation of  $\beta$ -[4(5)-imidazolyl]methylidene malonic acid

<sup>1</sup> Details concerning the preparation and purification of histidine and urocanic acid are available in the doctoral dissertation of W. J. Darby, on file in the Library of the University of Michigan, and available in microfilm.

in pyridine (16). This method of preparation is somewhat more convenient, particularly when the intermediate, 4(5)-hydroxymethylimidazole, is obtained by the procedure recently reported (17).

Samples of urocanic acid prepared by the two methods gave identical qualitative reactions and showed satisfactory purity on analysis. Urocanic acid synthesized by the second procedure gave the values presented in Table I on analysis. The melting point of urocanic acid varies markedly with the rate of heating (13). A preparation made by the first procedure melted<sup>2</sup> at 226–229° when heated rapidly, and at 222–222.5° when heated slowly. It melted simultaneously with a synthetic sample generously furnished by Dr. W. C. Rose, and a mixture of the two did not lower the melting point. In the same bath, the samples prepared by the two procedures outlined melted simultaneously at 223.5–224.5°. Since many imidazolyl derivatives melt within the range of 200–235°, it should be

TABLE I

*Analysis of Synthetic Urocanic Acid and of Urocanic Acid Isolated from Rabbit Urine*  
All analyses were carried out on the anhydrous acid.

Source of sample	Carbon	Hydrogen	Nitrogen
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Synthetic.....	52.08	4.22	20.03 (micro-Kjeldahl)
Experiment 2-1*...			19.80 "
" 6-8 ..	51.80	4.13	20.23 (micro-Dumas)
" 16-10	52.08	4.42	20.33 "
Theoretical .....	52.15	4.38	20.29

\* The water of crystallization as determined with the crystalline material was 20.7 per cent (theory, 20.68 per cent).

emphasized that for purposes of identification it is imperative that the melting points of an unknown compound and of a sample of urocanic acid of known purity and of a mixture of the two should be determined simultaneously. Similar precautions are necessary in the determination of melting points of the picrate and nitrate, derivatives commonly used in the identification of urocanic acid (6, 13). In our experience, the picrate has melted with decomposition between 220–225°, usually at 224–225°, and the nitrate within a range of 207–214°, usually at 212°. The melting points of all samples of urocanic acid and its salts described subsequently were determined under the conditions described above.

An attempt was also made to prepare urocanic acid by the prolonged action of *Bacillus paratyphosus* A on *l*-histidine (6). The isolation of

<sup>2</sup> Urocanic acid melts with decomposition. All melting points here reported are uncorrected.

urocanic acid was carried out as described subsequently for urine. Four experiments were carried out with two strains of the organism, obtained through the courtesy of Professor M. H. Soule and Dr. Marshall Snyder of the Department of Bacteriology. From one of the four flasks, each of which contained 2.7 gm. of *l*-histidine monohydrochloride and was incubated at 37° for 46 days, were obtained 36 mg. of a compound which gave all the reactions of urocanic acid and which melted at 224.5-225°. From a second similar culture, 2.0 gm. of material identified as histidine monohydrochloride by reactions and melting point were isolated, but no urocanic acid was obtained. It was obvious that the procedure was not suitable as a means of preparation of urocanic acid and further attempts were abandoned. It is of interest, however, that for the first time, so far as we are aware, the observations of Raistrick were confirmed, although the yield was much lower than those obtained by him.

*Isolation of Urocanic Acid from Urine*—No quantitative method for the determination of urocanic acid is available. In earlier studies, isolation has been attempted by a variety of procedures (1, 9, 11). No careful study of the recovery of urocanic acid added to normal urine appears to have been made. We have added 100 mg. of synthetic urocanic acid to a 24 hour specimen of rabbit urine and have not been able to recover any urocanic acid by the procedures described in the literature. The method which has proved most successful depends upon the precipitation of the sparingly soluble complex of silver and urocanic acid at a pH of 6.8 to 7.0 (3), a precipitate which is soluble in an excess of either ammonia or acid. The details of the isolation are as follows:

The volume of urine was reduced under diminished pressure to approximately 100 cc., acidified to Congo red with nitric acid, and a 50 per cent solution of silver nitrate added in excess. (Congo red is not reliable as an indicator in the presence of the silver ion.) After the material had stood an hour or so, the resulting precipitate was filtered off on a Buchner funnel, fitted with a thin layer of Filter Cel, and extracted three times with hot water which had been acidified with a few drops of 10 per cent nitric acid. The combined filtrate and washings were brought to a pH of 6.8 to 7.0 (phenol red) by the careful addition of 10 per cent ammonium hydroxide solution, allowed to stand a few hours, and filtered. A few cc. of the silver nitrate solution were added to the clear yellow filtrate to insure an excess of this reagent, and, if necessary, the pH of the solution was readjusted to 6.8 to 7.0. Any additional precipitate which formed was removed and added to the silver precipitate.

The moist tan precipitate was washed with two portions of water, suspended in about 75 cc. of water, and the silver was precipitated by hydrogen sulfide. The sulfide was filtered off and extracted three times with hot

water. The combined clear filtrate and washings were evaporated to dryness under reduced pressure at a temperature of less than 60°. The residue was taken up in a minimum amount (less than 10 cc. total volume after being filtered and washed) of boiling water and filtered. Dilute ammonium hydroxide was then added dropwise to the clear brown solution with cooling until it was very faintly acid to Congo red paper and the material was allowed to stand overnight in the refrigerator. If the solution were alkaline rather than acid, dilute hydrochloric acid was added rather than ammonium hydroxide.

The tan precipitate which formed was filtered off, dissolved in a minimum quantity of boiling water, and filtered through a thin pad of carbox E. The filtrate was chilled overnight in the refrigerator and the white shimmering needles of urocanic acid were filtered off, dried over calcium chloride, and weighed. A second recrystallization was usually unnecessary. The pH of the mother liquor was adjusted with the glass electrode to  $4.6 \pm 0.2$ . Any further crystals which deposited when the material stood in the re-

TABLE II  
*Recovery of Urocanic Acid Added to Urine of Control Rabbits*

Added	Recovered	
mg.	mg.	per cent
96	42	43
120	61	51
200	120	60
500	313	63

frigerator were removed and recrystallized. The mother liquors were reserved for attempts to isolate other imidazole derivatives.

No failures were experienced when this method was applied to urines known to contain urocanic acid. The results of some recovery experiments in which the indicated amounts of urocanic acid<sup>3</sup> were added to the 2 day pooled urine samples of a rabbit are shown in Table II. The recovery of urocanic acid by this method was from 43 to 63 per cent with an average of about 55 per cent. The use of this factor serves to give a rough approximation of the quantity present in a sample of urine. The minimum amount of urocanic acid which could be detected was not determined. However, we believe that a failure to isolate the acid from urine by this method indicates that less than 100 mg. was present.

Despite the use of a variety of procedures in attempts to isolate other

<sup>3</sup> Urocanic acid as ordinarily obtained contains 1 molecule of water of crystallization. All yields of isolated material reported in Tables II to IV are of the crystalline acid.



imidazole derivatives from the mother liquors from the urocanic acid, histidine alone was found. The most efficient method for the isolation of histidine from these solutions consisted of diluting to a volume of 100 cc., adding 30 cc. of a saturated alcoholic solution of mercuric chloride, and neutralizing to litmus with sodium carbonate solution. The resulting

TABLE III

*Imidazole Derivatives Isolated from Rabbit Urine Following Administration of Histidine*

Except in Experiment 9-6, histidine monohydrochloride was administered; in this experiment, the dihydrochloride was fed.

Experiment No.*	Salt administered†	Mode of administration	Imidazoles isolated‡		Remarks
			Urocanic acid	Histidine HCl	
	gm.		mg.	mg.	
1-2	15 (3 × 5)	Oral	251	0	Death after 3rd dose
2-1	15 (3 × 5)	"	1118	0	" " 3rd "
3-3	5 (1 × 5)	"	73	0	Signs of toxicity; delayed paralysis
4-6	5 (1 × 5)	"	0	192	No signs of toxicity
5-7	10 (2 × 5)	"	0	589	" " " "
6-8	5 (1 × 5)	"	73	219	Signs of toxicity; died
7-7	10 (2 × 5)	Subcutaneous	0	371	No signs of toxicity
8-7	5 (1 × 5)	Oral	0	§	" " " "
9-6	12 (2 × 6)	"	50	§	Signs of toxicity; died
10-9	10 (2 × 5)	Subcutaneous	0	2003	No signs of toxicity
11-9	10 (2 × 5)	Oral, subcutaneous	0	§	" " " "
12-10	10 (2 × 5)	Subcutaneous, oral	0	1360	" " " "

\* The second figure indicates the rabbit number.

† The figures in parentheses indicate the period of dosage in days and the amount administered each day, respectively. In Experiments 11-9 and 12-10, the histidine was administered orally and subcutaneously on 2 successive days as indicated.

‡ The weight recorded is that of the substance after recrystallization until a constant, acceptable melting point was reached.

§ No attempt at isolation was made.

white precipitate was allowed to stand a few hours, filtered, washed, suspended in 125 cc. of distilled water, and decomposed with hydrogen sulfide. The remainder of the isolation was conducted essentially as described by Foster and Shemin (14), but on a proportionately reduced scale. The amino acid salt was usually pure after one recrystallization.

Male rabbits of 1.7 to 2.85 kilos of body weight were fed oats and

cabbage, in amounts sufficient to maintain the weight of the animals. The *l*-histidine monohydrochloride was dissolved in warm water (usually 5 gm. in 25 cc.) and partially neutralized by the addition of sodium carbonate (0.6 gm.). This solution was administered either by stomach tube or by subcutaneous injection as indicated in Table III. When more than one dose was given during the experiment, the doses were administered on consecutive days. The urine was collected throughout the experimental period and during the 24 hours following the last dose of histidine, unless death supervened. The experimental samples were subjected to the above procedure for the isolation of urocanic acid. The normal urines of Rabbits 1 to 5 and Rabbit 13 were examined for the presence of urocanic acid. None was detected. Prior to each experiment the Pauly reaction was applied to the urine of each animal. In no case was a marked red color observed; hence it was concluded that none of the rabbits was excreting abnormal amounts of imidazole compounds.

Synthetic urocanic acid was similarly administered either orally or subcutaneously to Rabbits 9, 10, and 13. The acid was neutralized to litmus by the addition of sodium hydroxide.

#### DISCUSSION

Urocanic acid was isolated from the urine of five of the eight animals to which histidine was administered (Table III). Its identity was established by the method of isolation, the qualitative reactions (3, 6), and the melting points of the free acid and its salts, the picrate and nitrate. The analyses of two samples of the anhydrous acid isolated from the urine in Experiments 2-1 and 6-8 are presented in Table I. The identity of the isolated urocanic acid appears to have been satisfactorily established.

It should be noted that urocanic acid was excreted by only those animals to which the histidine was administered orally. No animal excreted urocanic acid in detectable amounts after the subcutaneous injection of histidine. These results might be interpreted to indicate that the excreted urocanic acid was formed within the gastrointestinal tract, possibly by the action of the bacterial flora, as suggested by Raistrick (6). Such an interpretation, however, is not to be reconciled with the reported isolation of urocanic acid from the urine of the rabbit following injections of histidine (11). In the present experiments, not all of the animals which were fed histidine excreted urocanic acid.

Another striking observation was the correlation between the excretion of urocanic acid and the appearance of signs of toxicity. Without exception those rabbits which excreted urocanic acid in the urine after the feeding of histidine exhibited a marked toxicity, characterized by anorexia, rapid heart rate, difficult respiration, paralysis of the posterior extremities, and,

in all but one case, death. The urines of these animals exhibited no abnormal pigmentation, no pathological reducing substances, and only traces of protein. Autopsy revealed an enlarged right heart and distended lungs. Microscopic examinations carried out by Dr. C. V. Weller of the Department of Pathology of the University of Michigan revealed hemorrhage by diapedesis, acute edema of the pulmonary tissue, contraction of the pulmonary arteries and of the smooth muscles of the bronchi, and an abundance of eosinophiles. No signs of toxicity appeared in the animals when the oral or subcutaneous administration of similar doses of histidine did not result in the excretion of urocanic acid. Kotake and Konishi (9) specifically state that none of the dogs fed histidine showed more than a transitory indisposition, but that an animal injected with histidine monohydrochloride did manifest symptoms of toxicity. These symptoms were not described. Kiyokawa (11) made no mention of the presence or absence of signs of toxicity in the rabbit following the injection of histidine hydrochloride. The authors in unpublished experiments have noted the appearance of a red pigment in the urine of rats fed large amounts of histidine, and Remmert and Butts (18) reported the appearance of hematuria in rats after the feeding of histidine. Åkerblom (19) has found that the symptoms of founder could be reproduced in horses by the oral administration of 10 gm. of histidine and cultures of certain strains of *Bacillus coli*, and attributed the observed signs to histamine formed within the gastrointestinal tract of the animals. Whatever the mechanism of the toxicity, the close association of the excretion of urocanic acid with the development of a severe toxic reaction indicated that some abnormal metabolic process was occurring within those animals producing urocanic acid. The complete absence of any such signs in the rabbits which did not produce urocanic acid, but did excrete histidine, would signify a more nearly physiological reaction.

Histidine was isolated in six of the nine experiments in which the urine was examined after the administration of this amino acid. The isolated histidine monohydrochloride was identified by qualitative tests, by the melting point of the pure substance, and by mixed melting points with a sample of known purity. The histidine monohydrochloride obtained in Experiment 6-8 contained 16.76 per cent of chlorine (theoretical, 16.91 per cent). A maximum of 5.9 per cent of the histidine administered was isolated from the urine after feeding, and from 3.7 to 20.0 per cent in the injection experiments. Both histidine and urocanic acid were isolated from the same urine in only one experiment.

In none of the previously reported studies of the formation of urocanic acid after the administration of *l*-histidine was the urinary excretion of histidine investigated. The excretion of small amounts of histidine by dogs

and guinea pigs following the administration of the amino acid has been observed. The data herein reported demonstrate that the rabbit, like the dog, may excrete part of the *l*-histidine monohydrochloride when administered either orally or subcutaneously. Histidine is not a constituent of normal rabbit urine (20).

The results of the experiments in which synthetic urocanic acid was administered are presented in Table IV. The identity of the isolated urocanic acid was established by the procedures already described. Analyses of the acid isolated in Experiment 16-10 are presented in Table I. These data demonstrate that urocanic acid is excreted unchanged by the rabbit, in contrast to certain other substituted acrylic acids which have been investigated (21). None of the animals which received urocanic acid manifested the slightest sign of toxicity. Hence, it is improbable that this

TABLE IV  
*Excretion of Urocanic Acid by Rabbit after Administration of the Acid*

Experiment No.	Acid administered	Mode of administration	Urocanic acid isolated
	gm.		mg.
13-9*	0.170	Oral	0
14-10	0.175	Subcutaneous	42
15-9	1.000 ( $2 \times 0.5$ )*	Oral	0
16-10	1.000 ( $2 \times 0.5$ )	Subcutaneous	394
17-13	3.000	Oral	126

\* For the explanation, see the foot-notes to Table III.

substance was the toxic agent responsible for the death of those animals which excreted urocanic acid following the administration of histidine.

These data establish that the failure of certain rabbits to excrete urocanic acid following the administration of histidine is not due to an inability of the rabbit to excrete the substance, or to a peculiar ability of the animal to destroy large amounts of urocanic acid. From the data on the fate of injected urocanic acid, it seems reasonable to conclude that the failure of certain rabbits to excrete urocanic acid, following the administration of large doses of histidine, was due to the failure of those animals to convert any considerable portion of the histidine into urocanic acid.

Konishi (22) has presented data purporting to show that when urocanic acid was fed to a fasting rabbit the added nitrogen appeared quantitatively in the urine. However, from his data it may be calculated that the extra urea nitrogen alone amounted to approximately  $1\frac{1}{2}$  times that of the nitrogen administered as urocanic acid. Furthermore, some urocanic acid was stated to have been isolated from the same urine. The efficacy of the

isolation procedure was not established; so the probable amount of urocanic acid excreted cannot be estimated. Edlbacher and Baur (23) have maintained that the guinea pig excretes urocanic acid following the administration of histidine. The present authors, in unpublished experiments, have been unable to obtain any evidence of this in similar experiments with these animals.

The data presented in this investigation demonstrate that urocanic acid is probably not the main pathway through which histidine is degraded. Thus, if a correction factor for the isolation of urocanic acid is applied, it may be estimated that from 40 to 70 per cent of the urocanic acid injected into Rabbit 10 was excreted in the urine. When 10 gm. of histidine monohydrochloride were injected into this same animal (Experiment 12-10), only 1.36 gm. of the salt of the amino acid were isolated from the urine. Assuming a loss of 50 per cent in the isolation of the histidine, the animal was able to destroy on a molecular basis 33 to 60 times more histidine monohydrochloride than urocanic acid. Furthermore, if the excretion of urocanic acid is a manifestation of the overproduction of a normal metabolite, it is difficult to explain why none of this compound could be isolated in the six experiments in which a total of 55 gm. of *l*-histidine monohydrochloride was administered to Rabbits 7, 9, and 10. The unflinching parallelism of toxicity and urocanic acid production by the rabbit would suggest that some abnormal metabolic process was occurring within those animals which produced urocanic acid. These considerations, in conjunction with the experiments on rats ((12, 13), and unpublished data of the authors), make it improbable that urocanic acid is a quantitatively important intermediate in the normal metabolism of histidine.

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#### SUMMARY

1. A dependable method for the isolation of urocanic acid from urine has been described.
2. The conversion of *l*-histidine into urocanic acid by *Bacillus paratyphosus* A (6) has been confirmed.
3. Five rabbits of a series of eight were found to excrete urocanic acid in the urine after the oral administration of large doses of *l*-histidine. No urocanic acid could be isolated from the urine of rabbits following the subcutaneous injection of *l*-histidine.

4. Severe toxic manifestations were exhibited by all of the rabbits which excreted urocanic acid following the administration of histidine. No such signs were exhibited by animals which received histidine and did not excrete urocanic acid or by rabbits to which synthetic urocanic acid was administered, orally or subcutaneously.

5. Rabbits excreted histidine in the urine following the oral or subcutaneous administration of that amino acid.

6. Rabbits excreted unchanged a considerable portion of the injected synthetic urocanic acid.

7. It was concluded that urocanic acid was not a quantitatively important intermediate in the normal metabolism of histidine.

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## ON LYSINE AND ORNITHINE

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The object of the following communication is to record certain observations on lysine and ornithine which may possibly be of some use to other workers with amino acids. The separation of lysine from histidine and arginine, based on the sparing solubility of the silver salts of the latter, was described by Kossel (1) in 1898 and has proved its value in the hands of countless workers. It has generally been assumed that lysine was not precipitated as a silver salt in either neutral or alkaline solution in the presence of excess of silver nitrate. Kossel after describing the precipitation of histidine and arginine with silver writes, "Macht man denselben Versuch mit Lysin, so bemerkt man entweder überhaupt keine Niederschlagbildung oder eine solche, welche sich leicht im Ueberschuss von Natron löst, oder nachdem mehr Silber hinzugefügt worden ist, eine Fällung von Silberoxyd. Es entsteht aber keine in Natron unlösliche weisse Silberverbindung des Lysins." It should be noted, however, that the concentration of the lysine solution used is not indicated. On the other hand in a later experiment in which the products of hydrolysis of 20 gm. of protamine are separated with silver sulfate he states that the volume should amount to at least 1500 cc.

On the other hand Hedin (2) some 3 years earlier described two silver salts of lysine, one of them an acid salt  $C_6H_{14}N_2O_2 \cdot HNO_3 \cdot AgNO_3$  which is a finely crystalline substance freely soluble in water. The other salt  $C_6H_{14}N_2O_2 \cdot AgNO_3$  had an alkaline reaction and was "in Wasser ziemlich schwerlöslich, aber bei weitem nicht in dem Grade wie das Argininsalz."

On several occasions recently the writer had occasion to use silver nitrate and sodium hydroxide as a precipitant in a mixture containing chiefly dibasic monoamino acids. Arginine had been previously removed as flavianate and the monoamino acids had been almost all removed by extraction of the slightly alkaline solution with butyl alcohol. The reagents were used in the fashion previously described by the writer (3) using alternately 15 per cent silver nitrate and  $N$  or  $2 N$  sodium hydroxide, until brown silver oxide began to appear mixed with the white silver salts. The latter were filtered off, washed thoroughly, and decomposed with hydrogen sulfide. Nitrate was invariably present to some extent, indicating the probable presence of a certain amount of complex  $AgNO_3$  salts in the silver precipitate. The bases precipitated by silver were precipitated with phos-



photungstic acid and regenerated in the usual fashion. Histidine was, as expected, present in significant amount and was separated from other bases by precipitation with mercuric sulfate. After removal of the excess of mercury and sulfates from the filtrate, it was found to contain large amounts of lysine and some ornithine which were thoroughly identified. In one experiment in which some 300 gm. of amino acids were present in the solution before precipitation with silver, in a volume of about 12 liters, no less than 45 gm. of recrystallized lysine picrate was isolated. The lysine picrate slowly darkened and decomposed above  $230^{\circ}$ , had the correct amount of amino nitrogen (7.4 per cent), and was converted into the dihydrochloride melting at  $193^{\circ}$  with a rotation of  $[\alpha]_D^{20} = +15.0^{\circ}$  in 5 per cent solution. The only variation observed from the accepted data was in the melting point of phenylhydantoin derivatives to which reference will be made later.

The preceding results would indicate that lysine and ornithine may be precipitated with silver to a greater extent than was generally recognized. Accordingly a number of experiments were made in which lysine or ornithine base, 0.3 gm., prepared either from pure picrate or hydrochloride was treated with silver nitrate and sodium hydroxide at varying dilutions. Excess of silver nitrate, about  $2\frac{1}{2}$  equivalents, was added by degrees and an equivalent amount of normal sodium hydroxide was added at intervals until a definite separation of brown silver oxide occurred. The resulting precipitate was filtered off, washed with ice-cold water, decomposed with hot dilute hydrochloric acid, and the precipitated base estimated by means of an amino nitrogen determination by Van Slyke's method. The results showed that in concentrations of ornithine and lysine varying from 0.15 to 1.0 per cent the silver precipitate contained from 5.0 to 9.3 per cent of the total base. These amounts are obviously sufficiently large to introduce serious error if unrecognized. Moreover there is good reason to believe that even larger amounts of ornithine and lysine may be precipitated when they are accompanied by other amino acids giving sparingly soluble silver salts.

The derivatives of ornithine and lysine, prepared by combination with 2 molecules of phenyl isocyanate and subsequent hydantoin ring closure on treatment with acid, are among the most useful substances for the identification of small quantities of these bases. Unfortunately, however, there is an almost complete lack of agreement as to the exact melting points of these derivatives. Herzog (4), at Kossel's suggestion, first prepared these derivatives and recorded uncorrected melting points of  $191-192^{\circ}$  for the compound from *D*-ornithine and  $183-184^{\circ}$  for that from *D*-lysine. It is significant that Herzog prepared his hydantoins by evaporation on the water bath of small amounts of the phenyl isocyanate compounds with 25

per cent hydrochloric acid, and makes the curious (and erroneous) statement that the reaction does not take place on boiling with hydrochloric acid under a reflux condenser. Kossel and Dakin (5) used the ornithine derivative for identifying the products of the action of arginase and recorded a melting point of 199–200° (uncorrected). Fischer and Weigert (6) gave the corrected melting points of the dextro and inactive lysine derivatives as 185° and 196° respectively. The hydantoins were prepared by boiling with 30 per cent hydrochloric acid for about a quarter of an hour and they state that longer heating leads to secondary decomposition. Sørensen (7) recorded identical melting points for the active and inactive derivatives of ornithine, namely 191° (corrected), while the derivatives of dextro and inactive lysine were reported as melting at 194–195° and 187° respectively.

TABLE I  
*Racemization and Depression of Melting Points of Hydantoins on Boiling with Acid*

Phenylhydantoin derived from	Time of boiling with acid	M. p. of crude product	M. p. of recrystallized product	Rotation, $[\alpha]_D^{20}$
	min.	°C.	°C.	degrees
d-Lysine.....	1	197–199	200–202	–62.5
“.....	2.5	200–202	200–202	–62.5
“.....	15	195–197	198–200	–43.5
“.....	45	188–190	192–193	–30.0
dl-Lysine.....	2.5	190–191	190–191	
d-Ornithine.....	2.5	205–206	208–209	–48.0
“.....	30	187–190	194–195	–14.5
dl-Ornithine.....	2.5	191–192	191–192	

Investigation showed that the cause of these varying melting points of substances of undoubted analytical purity lay in the fact that the hot hydrochloric acid used in the conversion of the phenyl isocyanate derivatives into the hydantoins causes a progressive racemization of the latter, so that the product from an optically active amino acid is liable to be heavily contaminated with the inactive form. The racemization of hydantoins by hydrochloric and other acids has been previously recorded by Dakin and Dudley (8). In order to convert the phenyl isocyanate derivative into the hydantoin as rapidly as possible, the addition of alcohol to the hydrochloric acid is desirable in order to effect prompt solution. It was found that a mixture of 65 cc. of concentrated hydrochloric acid, sp. gr. 1.19, and 35 cc. of alcohol served well and that hydantoin formation was complete after 2½ minutes boiling, 10 cc. of the acid mixture being used for each 0.5 gm. of phenyl isocyanate derivative. On dilution with water the hydan-

toin separates almost quantitatively and is best recrystallized from diluted acetic acid rather than the customary alcohol. By means of the above technique the following uncorrected melting points were obtained with a small bulbéd calibrated thermometer: phenylhydantoin derivatives from *d*-ornithine 208–209°, inactive ornithine 191–192°, *d*-lysine 200–202°, inactive lysine 190–191°. The optical rotation of the two active derivatives was observed in 2 per cent solution in pyridine and amounted to  $[\alpha]_D^{20} = -48.0^\circ$  for ornithine and  $[\alpha]_D^{20} = -62.5^\circ$  for lysine. For obvious reasons these must be regarded as minimal values, since the possibility of some racemization cannot be excluded. Table I illustrates the progressive racemization of the hydantoins on prolonged boiling with acid and the resulting depression of melting points. It may in conclusion be noted that a partially racemized hydantoin may be completely racemized by dissolving it in cold 0.5 *N* sodium hydroxide. After an interval of 24 hours all activity is lost and the homogeneous hydantoin may be precipitated on acidification. For purposes of identification this procedure may be of considerable utility.

#### SUMMARY

It is shown that significant amounts of ornithine and lysine may be precipitated by silver nitrate and sodium hydroxide.

The formation of phenylhydantoins from phenyl isocyanate derivatives of ornithine and lysine by the action of acid is shown to be accompanied by racemization. Conditions are indicated by which racemization may be limited and optically homogeneous products obtained having definite melting points.

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## VITAMIN INTERRELATIONSHIPS

### II. THIAMINE AND RIBOFLAVIN INTERRELATIONSHIPS IN METABOLISM\*

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In Paper I of this series (1) results were reported on the influence of vitamin A deficiency and of a deficiency of various components of the vitamin B complex on the ascorbic acid content of various organs and endocrine glands. It was found that in vitamin A deficiency there was a reduction in ascorbic acid of the heart, kidney, and thymus. In multiple vitamin A depletions there were also losses of ascorbic acid from the adrenals, thyroids, and pituitary. In thiamine deficiency there were significant losses of vitamin C in the lung, kidney, and liver. Repeated thiamine depletions produced additional heavy losses of vitamin C in the kidney, liver, and thymus. In riboflavin deficiency the greatest reduction in ascorbic acid was found in single vitamin depletions. The losses were largely from the liver, kidney, lung, thymus, and thyroids. In pyridoxine deficiency no noteworthy changes occurred in ascorbic acid content of either tissues or glands.

Morgan (2) reported that the administration of nicotinic acid or pantothenic acid alone to dogs receiving ample amounts of all necessary vitamins except those of the "filtrate factors" resulted in their gradual loss of neuromuscular control and sometimes sudden death. Recently Supplee and coworkers (3) found that thiamine and pantothenic acid deficiencies interfere with mobilization of riboflavin in the liver during digestion and assimilation. The influence, however, of pyridoxine deficiency on the riboflavin metabolism of the liver was very slight.

#### EXPERIMENTAL

Because of the increasing interest in thiamine and riboflavin as oxidative catalysts, a study was undertaken of the possible interrelationship of these vitamins in metabolism. Since such an investigation involved frequent analyses of urine and feces as well as numerous determinations of various tissues and endocrine glands, rapid and reliable methods were necessary for this type of research. For thiamine we found the method of Hennessy and Cerecedo (4), as modified by the staff of the research laboratories

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of Merck and Company, Inc., (5) quite satisfactory. For the riboflavin content of feces, tissues, and glands we followed the procedures of Conner and Straub (6). For the riboflavin content of rat urine we used our modification of the method of Hodson and Norris (7) for determining the riboflavin content of foodstuffs, details of which will be given later.

With the exception of one group of eight animals, all the experiments were conducted by the paired feeding technique previously used in this laboratory (8); *i.e.*, the control animals were restricted to the same amount of food as was consumed by the pathological litter mates of the same sex. Consequently, the plane of nutrition was eliminated as a possible complicating factor in the study. The following is the composition of the basal ration used for the production of thiamine and riboflavin deficiencies: casein (purified),<sup>1</sup> 20; agar-agar, 2; Sure's Salts 1, 4 (9); butter fat, 10; and cerelose, 64. This ration<sup>2</sup> was supplemented daily with 20  $\gamma$  of thiamine, 20  $\gamma$  of riboflavin, 20  $\gamma$  of pyridoxine, 6 mg. of choline chloride, and 200  $\gamma$  of calcium pantothenate. In the experiments on thiamine deficiency no vitamin B<sub>1</sub> was allowed the avitaminotic rats and in those on riboflavin deficiency none of the latter vitamin was given the pathological animals. Following 2 weeks of thiamine depletion, the avitaminotic rats began to lose weight and generally succumbed 3 to 4 weeks thereafter, the maximum period of experimentation, therefore, being 6 weeks. The results on thirty-two pairs of animals, nineteen pairs of males and thirteen pairs of females, show an average loss of weight per animal during the vitamin depletion periods of 45.7 gm. for the thiamine-deficient rats and a loss of 31.4 gm. per animal for the controls on the same daily food intake, which substantiates our previous conclusions that there is a pronounced tissue catabolism in thiamine deficiency (8).

Riboflavin deficiency was produced in twenty-two pairs of animals, the depletion periods lasting 55 to 100 days. The symptomatology observed was the same as previously reported (10); *i.e.*, rough hair, alopecia, keratitis, conjunctivitis, and premature senility. In addition, we observed that some of the riboflavin-deficient animals, after 5 to 6 weeks depletion, developed "blood-caked" whiskers, reported by Unna (11) to be present in pantothenic acid deficiency. The changes in body weight per animal during the entire vitamin depletion periods were as follows: riboflavin-deficient, -22.5 gm.; restricted controls, +36.5 gm.

*Procedures for Thiamine and Riboflavin Determinations in Urine*—The urine was collected in amber bottles containing 10 drops of toluene and 0.5 per cent chlorobutanol solution (6), adjusted to a pH of about 1.0 with normal sulfuric acid. The latter was added by being poured over the

<sup>1</sup> Supplied by The Borden Company, New York, under the trade name of Labco.

<sup>2</sup> To prevent development of rancidity, this ration was compounded twice weekly.

filter paper in the bottom of the large funnels placed in metabolism cages, a description of which was given in a recent publication (12). In the morning the funnels were washed with an additional 7 cc. of the chlorobutanol solution. The diluted urine was then filtered in 100 cc. graduated cylinders and was brought up to any convenient volume, 20 cc. being that generally used. The urine was now divided for thiamine and riboflavin determinations. Two 5 cc. portions were pipetted into 50 cc. Erlenmeyer flasks for riboflavin determinations and the remaining 10 cc. portion was used for the thiamine determination.

*Thiamine Procedure*—The diluted urine sample was adjusted to a pH of approximately 4.2 with sodium acetate buffer with brom-cresol green as an outside indicator. After the acidity of the urine was adjusted to the desired pH, the urine was transferred from the cylinder to a base exchange tube containing 750 mg. of purified Decalso. From then on the thiamine determination in urine was carried out by the modified procedures described by Merck and Company (5). We used the Pfaltz and Bauer fluorophotometer for both the thiamine and riboflavin determinations. The direct determination of thiamine in urine without adsorption (13) proved undependable in our experience.

*Riboflavin Procedure*—We found by numerous trials that the riboflavin content of rat urine could be determined with the same degree of accuracy when the adsorption on florisol was omitted, which at the same time dispensed with the step of elution with dilute pyridine. 95 to 100 per cent recoveries of riboflavin added to dilute urine were obtained by the following procedure and the results checked with those obtained by adsorption on florisol (6). Our procedure depends on the principle that riboflavin is reduced to a non-fluorescent form by sodium hydrosulfite; so that after such treatment the fluorescence, emanating from impurities or foreign substances in the urine, constitutes the blank. The riboflavin content of the urine is then obtained by subtracting the fluorophotometer reading after reduction from the initial reading before treatment with sodium hydrosulfite and sodium bicarbonate. The following is the technique we used: To 5 cc. samples of urine were added 5 drops of 4 per cent  $\text{KMnO}_4$  solution and 2 drops of glacial acetic acid. The mixture was then shaken vigorously for 2 minutes. 5 drops of 3 per cent  $\text{H}_2\text{O}_2$  solution were then added and the mixture was again shaken until all signs of excess  $\text{KMnO}_4$  and  $\text{H}_2\text{O}_2$  disappeared. Enough distilled water was now added to bring the volume up to 15 cc. Readings were then made in the fluorophotometer which was adjusted so that 0.05  $\gamma$  of riboflavin per cc. (0.75  $\gamma$  in 15 cc. of water) gave a galvanometer deflection of 20. After this reading was obtained, 0.2 to 0.4 cc. of a solution of 1 gm. of sodium bicarbonate and 1 gm. of sodium hydrosulfite in 20 cc. of distilled water, kept cool in an ice

bath, was added and another reading was taken. The difference in the two readings gave the fluorescence due to riboflavin. The final reading was converted to micrograms by means of a graph constructed by plotting micrograms of riboflavin *versus* galvanometer readings. Since the curve is linear, even as low a reading as 0.013  $\gamma$  per cc. is quite accurate. The amount of riboflavin in the sample is as follows: The reading from the graph  $\times 15 \times ((\text{total volume of urine})/(\text{volume used for riboflavin determination}))$  equals micrograms of riboflavin.

*Thiamine and Riboflavin Determinations in Animal Tissues*—The various tissues or organs were pooled from groups of five to six pairs of rats, equal numbers from avitaminotic and control animals. They were covered with 50 per cent ethanol after being cut in thin slices and were dried at 55° overnight. They were then extracted thoroughly with petroleum ether in Soxhlet extractors, finely ground, and analyzed according to the procedures given above. The petroleum ether extracts of the tissues were found to be entirely free from thiamine and riboflavin. Since at 55° 6 to 8 per cent moisture was often left in the dried tissues, moisture determinations were made at 103° and all results were expressed on a moisture-free basis.

Equal amounts of samples were taken of the avitaminotic and control animal tissues. To the tissues were added 0.1 N sulfuric acid. If the samples weighed 800 mg. or more, 75 cc. of acid were added, but if the samples weighed less than 800 mg., 50 cc. were taken. The following volumes of acid were used for the glands pooled from thirty-two animals, for the thymus and adrenals 50 cc., and for the thyroids and pituitary 25 cc. The tissues were autoclaved for 20 minutes at 15 pounds pressure, cooled, the pH adjusted to 4.0 to 4.5, 5 cc. of 10 per cent taka-diastase were added, and the solutions incubated for 2 hours at 45°. After incubation the pH occasionally changed and required readjustment. The solutions were transferred to 100 cc. volumetric flasks and brought up to volume. They were then centrifuged until clear. Aliquots were taken for thiamine and riboflavin determinations and the procedures of Conner and Straub (6) were then followed. We were able to recover 90 to 100 per cent of the thiamine and riboflavin added to autoclaved extracts of the various animal tissues.

*Thiamine and Riboflavin Determinations in Feces*—The thiamine determination of feces was satisfactorily carried out according to the technique adopted for the tissues. The riboflavin determination of feces, however, presented considerable difficulties. The results of the first few weeks were very erratic, frequently the weekly fecal excretion being twice the total intake. The high figures were most probably due to bacterial synthesis, such as Lamoreux and Schumacher (14) observed in the case of the fowl.

Bacterial synthesis in the feces of the rat was recently reported by Wildemann (15). Following numerous failures we finally were able to reduce the riboflavin excretions in the rat feces to what appeared to be reasonable figures. The change responsible for reducing the apparent bacterial synthesis to insignificant proportions consisted in the collection of the feces under petroleum ether, the solvent used in the next step for removing fats previous to analysis. Feces were collected daily from each animal in the metabolism cages in amber bottles which were filled with enough petroleum ether to cover the feces. At the end of the metabolism period, usually a week, the feces were removed from the bottles and were placed in evaporating dishes in the dark room to dry. After drying, the feces were pulverized in a mortar and fat was extracted with petroleum ether overnight in Soxhlet extractors. The petroleum ether extracts were free from thiamine and riboflavin. After fat extraction they were analyzed by the same procedures as the tissues, the additional precaution taken being that the extracts of feces were filtered hot to prevent solidification.

#### *Thiamine and Riboflavin Interrelationships in Metabolism*

*Thiamine Deficiency*—The riboflavin and thiamine excretions in the urine in thiamine deficiency were studied in thirty-five pairs of rats. Four pairs of animals received food *ad libitum*, while the food of the control rats of the rest of the groups was restricted to that consumed by the avitaminotic animals. The metabolism periods ranged from 7 to 14 days. The urine was analyzed daily with the exception of Sundays. Monday's sample, therefore, covered a 48 hour period. During thiamine depletions we observed not only a marked reduction of thiamine excretion in the urine but also a marked disturbance in riboflavin metabolism, as evidenced by urinary excretions. The increased riboflavin excretions in individual cases of thiamine-deficient animals varied from 2- to 7-fold compared with the excretions of the controls. The average weekly riboflavin excretions during the avitaminotic period for five groups of rats, comprising twenty-four pairs, were as follows: pathological, or thiamine-deficient, 92.5  $\gamma$ ; restricted controls, 28.3  $\gamma$ . The average weekly thiamine excretions were 1.35  $\gamma$  for the thiamine-deficient animals and 3.84  $\gamma$  for the restricted controls. No noteworthy differences were found in fecal excretions between pathological and control animals. These figures, however, were essential in calculating efficiency of riboflavin and thiamine utilization, the results of which are submitted in Tables I and II.

In Table III are presented summarized results on the influence of thiamine deficiency on the thiamine and riboflavin content of various tissues and endocrine glands. The data represent average figures on thirty-two pairs of animals, nineteen pairs of males and thirteen pairs of females.



While the various organs or tissues were analyzed from groups of five to six pairs of animals, the results of which were then averaged, the analyses

TABLE I  
*Influence of Thiamine Deficiency on Efficiency of Riboflavin Utilization*

Group No.	Pair Nos.	Animals*	Weekly riboflavin intake	Weekly fecal excretion	Digestible riboflavin	Weekly urinary excretion	Amount of riboflavin absorbed	Riboflavin utilized
			γ	γ	γ	γ	γ	per cent
3	12-17	P	140	26.25	113.75	146.98	-33.23	
		RC	140	31.38	108.62	41.86	66.76	61.5
4	18-23	P	140	27.92	112.08	98.76	13.32	11.8
		RC	140	41.00	99.00	21.44	77.56	78.4
5	24-29	P	140	12.81	127.19	65.02	62.17	48.1
		RC	140	28.13	111.87	32.22	79.65	71.1
Average		P		22.33	117.67	103.59	14.08	11.2
		RC		33.50	106.55	31.84	74.71	70.1
2†	7-11	P	350	38.25	311.75	205.16	106.59	34.2
		RC	350	38.20	291.80	91.06	197.74	67.7

\* P = pathological; RC = restricted control.

† Group 2 was not included in the average, since each animal instead of receiving the regular 20  $\gamma$  daily doses of thiamine, riboflavin, and pyridoxine received 50  $\gamma$  daily of these vitamins.

TABLE II  
*Influence of Riboflavin Deficiency on Efficiency of Thiamine Utilization*  
The weekly intake of thiamine was 140  $\gamma$ .

Group No.	Pair Nos.	Animals*	Weekly fecal excretion	Digestible thiamine	Weekly urinary excretion	Amount of thiamine absorbed	Thiamine utilized
			$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent
1	1-5	P	14.94	125.06	2.66	122.40	97.7
		RC	14.58	125.42	2.38	123.04	98.9
2	6-11	P	9.50	130.50	2.65	127.85	97.9
		RC	9.25	130.75	2.72	128.03	97.9
3	12-17	P	9.93	130.07	2.62	127.45	96.9
		RC	10.18	129.82	2.32	127.50	98.2
4	18-22	P	6.99	143.01	6.28	136.73	95.5
		RC	2.91	137.09	5.07	132.02	96.2

\* P = pathological; RC = restricted control.

on the glands were carried out on the pooled material collected from all the thirty-two pairs of animals, in order to have a sufficiency for thiamine and riboflavin determinations.

That there is a marked reduction in thiamine content of all the organs and endocrines in thiamine deficiency is quite evident, the greatest losses of vitamin occurring in liver, kidney, heart, and thymus. There are, however, also appreciable losses of riboflavin from the lung, ovaries, and muscles, and small losses from the rest of the body tissues in thiamine avitaminosis. The losses from the muscles, although not pronounced, are most significant, because they represent the largest weight of the carcass of the

TABLE III

*Influence of Thiamine Deficiency on Thiamine and Riboflavin Content of Various Tissues and Endocrine Glands*

The data were obtained from thirty-two pairs of animals, nineteen pairs of males and thirteen pairs of females.

Tissues	Thiamine		Change in patho-logical animals	Riboflavin		Change in patho-logical animals
	Patho-logical	Control		Patho-logical	Control	
	$\gamma$ per gm.	$\gamma$ per gm.	per cent	$\gamma$ per gm.	$\gamma$ per gm.	per cent
Liver.....	1.55	6.90	-77.5	36.70	41.28	-11.1
Kidney.....	1.69	8.28	-79.6	43.43	48.75	-10.9
Spleen.....	2.52	6.60	-61.8	15.11	16.10	-6.1
Heart.....	2.16	9.48	-77.2	34.43	39.13	-12.0
Lung.....	1.56	3.76	-58.5	8.81	10.39	-15.2
Brain.....	3.95	6.89	-42.4	7.59	8.13	-6.6
Testes.....	5.25	10.63	-50.6	5.45	5.64	-3.3
Ovaries.....	4.09	8.92	-54.2	22.55	29.65	-23.9
Stomach.....	1.41	3.50	-59.7	13.02	13.73	-5.2
Small intestines.....	0.81	2.08	-61.1	8.27	9.23	-10.4
Large ".....	0.83	2.20	-62.3	7.77	8.99	-13.6
Pancreas.....	1.01	2.45	-58.8	14.44	15.94	-9.6
Muscles.....	0.23	0.62	-62.9	1.62	2.01	-19.4
Thymus.....	4.00	19.00	-78.9	11.50	12.64	-9.1
Thyroids.....	5.00	8.00	-37.5	33.33	34.72	-4.0
Adrenals.....	9.00	16.00	-43.8	31.25	35.62	-12.3
Pituitary.....	4.50	6.00	-25.0	56.82	80.60	-29.6

animal. In sampling for muscle tissue, all the muscles were dissected out, dried, and analyzed as previously described. Therefore, the results are quite representative for the entire animal rather than portions of the various animals used in this study.

The approximate total weights of the glands were as follows: Thymus 270 mg., adrenals 200 mg., thyroids 75 mg., and pituitary 32 mg. The total weight of the thymus glands from the control animals was considerably more but the amount sampled was 270 mg., since this was the total weight of the thymus glands of the avitaminotic rats. While the total

weights seem too small for dependable results, the actual total amounts of riboflavin measured ranged between 0.113 and 1.313  $\gamma$ , which can be measured accurately in the Pfaltz and Bauer instrument. Therefore, the loss of about 30 per cent riboflavin in the pituitary in thiamine deficiency seems significant. The thiamine values in the glands, however, ranged between 0.13 and 0.6  $\gamma$ . The modified Hennessy and Cerecedo procedures (5) call for the determination of approximately 1.0  $\gamma$  of thiamine for the greatest accuracy. While this was possible for some of the tissues, such as muscle, liver, and kidney, in many tissues we were obliged to read less than 1.0  $\gamma$  and in urines of thiamine-deficient animals we frequently found only 0.2  $\gamma$  of thiamine. The results, however, while not quantitative in the cases of low readings, show a relationship between avitaminotic and control animals. Nevertheless, we are convinced that the endocrine glands of the rat, particularly the thyroid and pituitary, need a more sensitive method, such as the microbiological method, for measurements of such minute amounts of vitamins. Our results may therefore be considered suggestive of what may be found with a more sensitive microtechnique.

Since both fecal and urinary excretions of riboflavin in thiamine deficiency are available for four groups of animals, it was possible to calculate the amounts of riboflavin that were digested and absorbed and the percentage that was utilized. Group 3 (Table I) showed a negative balance, while the thiamine-deficient animals of Groups 2, 4, and 5 showed tremendous reduction in efficiency of riboflavin utilization compared with the control animals that received 20 to 50  $\gamma$  of thiamine daily. The average efficiency of riboflavin utilization for Groups 3, 4, and 5 was as follows: thiamine-deficient 11.2 per cent, restricted controls 70.1 per cent. It is clear from Table I that the greater losses of riboflavin in thiamine deficiency are due mainly to poor absorption. In the case of negative riboflavin balances, some of the riboflavin excreted in the urine undoubtedly originated from losses from the tissues, particularly the muscles.

*Riboflavin Deficiency*—The urinary and fecal excretions of thiamine and riboflavin in riboflavin deficiency were studied in twenty-two pairs of male rats (four groups) during vitamin depletion periods ranging from 31 to 78 days. The animals were, however, sacrificed for their tissues during advanced to terminal stages of deficiency which covered periods ranging from 55 to 100 days. The thiamine and riboflavin content of the various tissues (Table IV), therefore, includes that of animals that were well depleted of riboflavin. Since twenty-two animals did not provide enough material for analyses of the endocrine glands, the latter were not included in this study. That there are marked reductions of riboflavin in all the body tissues in riboflavin deficiency is evident from Table IV. The relatively smaller

losses of riboflavin from the body tissues in riboflavin avitaminosis than the losses of thiamine from the animal organs in thiamine deficiency are due to the fact that the animals in the latter avitaminosis were in a more advanced vitamin-depleted state than the animals in riboflavin deficiency. It is clear from Table II that even in advanced states of riboflavin deficiency, characterized by losses of body weight and general symptomatology, there is absolutely no disturbance in thiamine metabolism.

The results of this investigation may have considerable human application. Since it is now generally recognized that thiamine deficiency is wide-spread in this country (16), it is quite possible that border line ribofla-

TABLE IV

*Influence of Riboflavin Deficiency on Riboflavin and Thiamine Content of Various Tissues*

The data were obtained from twenty-two pairs of male rats.

Tissues	Riboflavin		Change in patho-logical animals	Thiamine		Change in patho-logical animals
	Patho-logical	Control		Patho-logical	Control	
	$\gamma$ per gm.	$\gamma$ per gm.	per cent	$\gamma$ per gm.	$\gamma$ per gm.	per cent
Liver.....	24.48	53.16	-54.0	5.81	6.27	-7.3
Kidney.....	45.74	74.69	-38.7	7.00	7.36	-4.9
Spleen.....	16.21	28.64	-43.4	5.37	5.19	+3.4
Heart.....	36.62	59.59	-38.5	9.60	10.34	-7.1
Lung.....	9.84	19.90	-50.6	3.55	3.68	-3.5
Brain.....	6.64	13.15	-49.5	5.91	6.21	-4.8
Testes.....	8.48	16.05	-47.2	23.31	22.40	+4.1
Stomach.....	6.85	16.56	-58.7	3.64	3.83	-4.9
Small intestines.....	7.16	13.16	-45.5	3.15	3.30	-4.6
Large ".....	6.69	13.94	-52.0	3.35	3.50	-4.3
Pancreas.....	9.60	19.78	-51.5	2.98	3.80	-21.6
Muscles.....	1.90	3.86	-50.0	0.59	0.67	-11.8

vin deficiencies may exist not only from inadequate riboflavin intake but also from poor utilization of the latter vitamin caused by thiamine deficiency. On the other hand, a diet abundant in thiamine may prevent riboflavin deficiency produced by insufficient riboflavin intake. These are, however, problems for the clinicians to solve.

## SUMMARY

In thiamine deficiency there is a pronounced disturbance in riboflavin metabolism, mainly because of poor absorption. However, in riboflavin deficiency there is no disturbance in thiamine metabolism.

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# STUDIES ON THE METABOLISM OF BRAIN SUSPENSIONS

## II. CARBOHYDRATE UTILIZATION\*

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In a previous paper (1) conditions which affect the respiration of brain preparations were described and it was shown that suspensions of brain tissue, prepared in isotonic medium under proper conditions, are perhaps more suitable than slices for the study of brain tissue metabolism and allow much more accurate sampling for gasometric and analytical studies.

A study has now been made of the extent to which the respiration of isotonic brain tissue suspensions can take place at the expense of glucose, lactate, or pyruvate. It is shown that brain tissue can metabolize non-carbohydrate materials, particularly when the normal substrate is lacking. Consumption of non-carbohydrate substances by the brain may be an important aspect of the insulin shock treatment for psychoses. Differences in behavior between glucose and lactate as substrates for brain tissue respiration have been noted. These differences may help to explain the observations by other workers that lactate is readily utilized by brain tissue *in vitro* and yet does not seem to support normal functions of the brain *in vivo*.

### Procedure

Whole rat brains were homogenized, by means of the instrument of Potter and Elvehjem (2), in warm Ringer-0.017 M phosphate solution (Krebs (3)) containing magnesium ions but with calcium omitted. Sufficient amounts of isotonic solutions of glucose (0.24 M), sodium pyruvate, or sodium *D*-lactate (0.13 M), dissolved in 0.017 M phosphate buffer containing 0.0012 M  $MgSO_4$ , were added to the tissue suspensions to give calculated initial concentrations of 0.15 per cent glucose or 0.2 per cent (0.022 M) pyruvic or lactic acid. The suspensions, after the additions were made, contained 150 mg. of fresh weight of tissue per cc.

Samples of the suspensions were pipetted into manometer flasks for gasometric measurements, and, for analytical measurements, similar samples were pipetted into spare manometer flasks, and all were shaken in the bath at 38°. Some of the analysis flasks were removed from the bath after 6½ minutes, just before the manometer taps were closed, others at

\* Presented in part before the American Society of Biological Chemists at Boston, April, 1942.

the end of the experimental period. The contents of the flasks were rapidly mixed and measured amounts were immediately pipetted into prepared vessels containing zinc sulfate solution, trichloroacetic acid, or sulfuric acid according to the determinations to be made.

For experiments on brains from insulinized animals, rats were fasted overnight and then received 3 units of insulin<sup>1</sup> intramuscularly. The animals were stuporous within an hour and within 2 hours some suffered convulsions. When four or five had had convulsions, they were decapitated for the experiment.

*Respiration Measurements*—For measurements of oxygen uptake, 3 cc. of suspension (450 mg. of tissue) were used in the flasks of Barcroft differential manometers. The gas space contained air, and CO<sub>2</sub> was absorbed by filter paper rolls soaked in KOH solution.

Respiratory quotients were determined according to the method of Elliott and Elliott (4). The validity of this method depends upon the assumption that the rate of oxygen uptake is the same in the absence of CO<sub>2</sub> (absorbed by alkali-soaked paper) as in the presence of CO<sub>2</sub> accumulating from the respiration. This assumption was shown to be valid for brain tissue. The oxygen uptake of a given suspension in the presence of alkali-soaked paper was found to agree exactly with that measured by the Dixon-Keilin apparatus in which CO<sub>2</sub> is not absorbed until after the end of the experimental period. Also, in the previous paper (1) it was shown that respiration occurred at the same rate in the presence or absence of added bicarbonate and 5 per cent CO<sub>2</sub>.

### *Analytical Methods*

*Glucose*—The tissue suspension, 7.5 cc., was pipetted into a 25 cc. volumetric flask containing 2.5 cc. of 10 per cent ZnSO<sub>4</sub>·7H<sub>2</sub>O and some water; 2.5 cc. of 0.5 N NaOH were immediately added with shaking and the volume was made up to 25 cc. with water.<sup>2</sup> Smaller samples, 5 or 6 cc., were sometimes taken with correspondingly smaller amounts of reagents. The fluid was centrifuged and 2 cc. or 1 cc. samples of the supernatant fluid were taken for duplicate determinations of reducing substances. Sometimes non-fermentable reducing substances were determined after yeast treatment.

The determinations were made by the method of Benedict except that, after the 6 minute heating with the copper reagent, the color reagent was added directly without cooling and the color was determined with the

<sup>1</sup> Aqueous solutions of amorphous insulin, kindly supplied by Eli Lilly and Company, were used.

<sup>2</sup> Protein precipitation with tungstic acid was not suitable, since high values are obtained if tungstate remains in the filtrate. It is difficult to adjust the amount of tungstate used so that it is exactly removed by the protein precipitate.

Klett-Summerson photoelectric colorimeter 20 minutes after addition of the color reagent and dilution (Elliott *et al.* (5)). Two standard glucose solutions of different strengths and a blank were run with every set of determinations and the glucose equivalents of reducing substances in the unknowns were read off from the standard curve.

*Total Carbohydrate*—Usually 3 cc. of the tissue suspension were pipetted into a 15 cc. centrifuge tube containing 2 cc. of 5 N  $\text{H}_2\text{SO}_4$  and 5 cc. of water. The tube was covered with a small funnel with a cut off stem; a thin glass rod passing through the funnel was used to stir the fluid occasionally. The tube was heated for 3 hours in a boiling water bath. The fluid was then neutralized to about pH 7 with 2 N NaOH from a burette, very small strips of Alkacid paper, handled with forceps, being used to test the pH. The neutralized fluid was introduced quantitatively into a 25 cc. volumetric flask by means of a dropping pipette; 1 cc. of 10 per cent  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  and 1 cc. of 0.5 N NaOH were added and the volume made up to 25 cc. The fluid was filtered and reducing substances were determined in duplicate before and after fermentation in the same way as for glucose. The recovery of standard glucose solutions treated with acid and heat was 96 to 97 per cent. In some cases the standards for total carbohydrate analysis were treated with acid and heat; in other cases the usual standards were used and the unknowns corrected by multiplying by 1.03.

98 to 100 per cent of glucose, 1.5 mg. per cc. of suspension, added to brain suspensions immediately before deproteinization with  $\text{Zn}(\text{OH})_2$  or treatment with acid, was recovered.

*Effect of Pyruvate on Glucose Determinations*—Pyruvate affects the Benedict determination appreciably. The effect of pyruvate decreases in a roughly linear manner with increase in the depth of color developed, whether the increased depth of color is due to glucose or to pyruvate itself. At low colorimeter readings, 1 mg. of pyruvic acid in the sample treated gives a color equivalent to that due to about 0.12 mg. of glucose; in the presence of 0.4 mg. of glucose, 1 mg. of pyruvic acid gives additional color equivalent to 0.06 mg. of glucose.

In experiments in which pyruvate was added as substrate, the apparent glucose and total carbohydrate values were corrected for the effect, at the particular colorimeter reading, of the amount of pyruvate present in the sample analyzed. The effect of pyruvate per mg. was estimated from an approximate curve of the pyruvate effect *versus* the colorimeter reading. After acid hydrolysis for the determination of total carbohydrate, the effect of pyruvate originally present was lower and the glucose equivalent was read from a different curve. The washed yeast used did not destroy pyruvate; so that the correction had to be applied also to the values for non-fermentable reducing substances.

*Lactic acid* was determined on deproteinized filtrates, after removal of



sugar with  $\text{CuSO}_4\text{-Ca(OH)}_2$ , by the method of Barker and Summerson (6). Either zinc hydroxide filtrates, obtained directly or after acid hydrolysis, or trichloroacetic acid filtrates could be used. The filtrates were so diluted that between 1 and 5  $\gamma$  of lactic acid were present in the final 1 cc. analyzed, since the color developed per unit of lactic acid often tended to fall off with amounts greater than 5  $\gamma$ . Duplicate determinations on the sugar-free fluid were always made and two standard lithium lactate solutions and a blank, treated with  $\text{CuSO}_4\text{-Ca(OH)}_2$ , were run with every set of determinations. 97 per cent of lactic acid, 1.5 mg. per cc. of suspension, added to brain suspension immediately before deproteinization, was recovered.

Barker and Summerson (6) found that pyruvic acid affects the lactic acid determination, 1  $\gamma$  of pyruvic acid giving a color equivalent to that resulting from 0.025  $\gamma$  of lactic acid, but that the  $\text{CuSO}_4\text{-Ca(OH)}_2$  treatment would remove up to 5  $\gamma$  of pyruvic acid per cc. of final solution analyzed. In a large number of trials with 5 to 30  $\gamma$  of pyruvic acid per cc. of final solution, in the presence or absence of lactic acid, we found the average color produced per microgram of pyruvic acid, at all concentrations of pyruvic acid, was equivalent to that given by 0.030  $\gamma$  of lactic acid, with small variations. There was no sign of any pyruvate removal by  $\text{CuSO}_4\text{-Ca(OH)}_2$ ; presumably our reagents or conditions varied slightly from those of Barker and Summerson. In experiments in which pyruvic acid was added, pyruvic acid was determined separately and appropriate corrections were applied to the apparent lactic acid values.

*Pyruvic Acid*—The suspension, 3 cc., was pipetted into 7 per cent trichloroacetic acid solution and made up to 10 or 25 cc. The filtrate was diluted as necessary and pyruvic acid was determined by the method of Lu (7) as modified by Bueding and Wortis (8). The range of the method is wider than was realized by the latter authors, since the color developed bears a straight line relationship to the pyruvic acid in the sample analyzed up to 40  $\gamma$ . Blanks were run with every set of determinations and, when large amounts of pyruvate were present, fresh standard sodium pyruvate solutions were also run. 95 per cent of pyruvic acid, 1.8 mg. per cc. of suspension, added immediately before deproteinization, was recovered. Probably small amounts were reduced to lactic acid or otherwise metabolized before the enzymes were inactivated. If the pyruvate was added after the protein precipitation, recovery was 100 per cent. Protein precipitation by  $\text{ZnSO}_4\text{-NaOH}$  often gave rather low recoveries of added pyruvate, but with the traces of pyruvate which are found when no pyruvate had been added to the tissue, either method of deproteinization seemed suitable.

*Acetic acid* was determined by steam distillation at atmospheric pressure by means of distilling apparatus with ground glass joints kindly loaned to us by Dr. W. C. Stadie. The determinations had to be made on tissue

filtrates containing lactate, pyruvate, and chloride. Lactic and pyruvic acids, particularly the latter, distil over appreciably and, if the pH of the solution is too low, HCl may also come over. By treating the solution with phenylhydrazine much of the pyruvic acid can be removed but this method did not completely prevent the effect of pyruvic acid on blank determinations. The most satisfactory separation of acetic acid from both pyruvic and lactic acids was achieved by distillation and redistillation before the final titration. By acidifying with  $\text{KH}_2\text{PO}_4$  for the first distillation, acetic acid distilled over without appreciable amounts of HCl coming over. A large amount of phosphate was used, since acetic acid distils more readily from strong salt solution (9). Care had to be taken to avoid overheating, since this always resulted in high blank determinations. This occurred if the fluid in the distilling flask was brought to boiling before steam was admitted. The determination was carried out as follows:

The tissue suspension was deproteinized with  $\text{ZnSO}_4\text{-NaOH}$  as for glucose determinations. A 4 or 5 cc. sample of the supernatant fluid was measured into the distillation flask and 2 gm. of solid  $\text{KH}_2\text{PO}_4$  were added. Steam was admitted to the distillation flask and then a heating coil was adjusted below so that the volume of fluid in the flask remained about 5 cc. 50 cc. of distillate were collected in an Erlenmeyer flask and made alkaline to brom-thymol blue with 0.005  $\times$  NaOH. The solution was boiled down to 2 to 3 cc. on an electric hot-plate; small beads were used to prevent sputtering. Care had to be taken not to allow the solution to evaporate dry; otherwise low values were obtained. The fluid was introduced quantitatively into the rinsed distilling flask to which 0.5 cc. of syrupy phosphoric acid was added. Steam distillation was carried out as before, 35 cc. of distillate being collected in a 50 cc. centrifuge tube. Nitrogen was bubbled through the distillate for 6 to 8 minutes and titration was then carried out with freshly standardized  $\text{CO}_2$ -free 0.005  $\times$  NaOH from a micro burette with brom-thymol blue as indicator, stirring being accomplished by continued bubbling. The distillation apparatus was steamed thoroughly before every distillation. The accidental presence of phosphate in the distillates was frequently tested for by the method of Feigl (10) but was found very rarely.

Complete blank determinations were carried out and the blank titration value, about 0.08 cc., was deducted from the values given by the tissue filtrates. The presence of 1.8 mg. each of pyruvic and lactic acids per cc. of suspension, giving about 2 mg. of each in the 4 cc. filtrate analyzed, scarcely affected the blank titration. 94 to 104 per cent of acetic acid, 0.25 to 0.5 mg. per cc. of suspension, added to brain suspension immediately before deproteinization and giving 0.3 to 0.6 mg. in the 4 cc. of filtrate analyzed, was recovered.

The method, of course, is not specific for acetic acid but we know of no

other volatile acids which would be produced in these experiments and affect the final titration very considerably. However, completely false values may be found if it is attempted to obtain larger titration values by taking larger samples, alkalizing, and evaporating them down to the 4 cc. required for distillation. Solutions containing small amounts of glucose, when heated in dilute alkali, give rise to appreciable amounts of acetic acid, as was proved by the high titration values and the lanthanum nitrate test of Krüger and Tehreich (10) applied to concentrated distillates.

### *Results*

In Table I the results of typical single experiments are presented. In Table II balance sheets have been drawn up from averages of several closely agreeing similar experiments to show the extent to which the gas exchange, measured manometrically, could be accounted for by the combustion or formation of the substances analyzed. When a substance, say glucose, disappears, the oxygen which would be absorbed in its complete combustion is entered as a negative quantity. When a substance like lactic acid is formed, presumably from the glucose, the oxygen which would be required for oxidation of the amount formed during the experimental period is entered as a positive quantity. Then the algebraic sum of the oxygen required for all the changes represents the actual gas usage possibly accounted for. It can be shown that this method of accounting also gives correct results in the more complicated situation when pyruvate is added and some of the pyruvate is completely oxidized, some is oxidized to acetate and  $\text{CO}_2$ , and some is reduced to lactate.

The balance sheets have to be interpreted with caution. There are undoubtedly changes, which have not been determined, in various intermediaries of carbohydrate metabolism, such as succinic and  $\alpha$ -ketoglutaric acids. However, with chopped rat brain Elliott and Greig (11) found only small accumulations of succinate from pyruvate and probably less would accumulate from other substances. Except when pyruvate and acetate were added to the suspensions, these substances were present only as traces, changes in which were small. It seems unlikely that large amounts of any single other intermediary product of glucose, lactate, or pyruvate metabolism would accumulate or disappear in a system free of special inhibitors, but the sum of small changes in a number of intermediaries not determined might be appreciable.

The oxygen uptakes with different substrates shown in Tables I and II are not directly comparable with each other, since they were obtained with suspensions from different rats. However, series of experiments in which the effects of the various substrates on samples of the same tissue suspension were compared gave results not materially different from the average values given in Table II.

Glucose—From studies of arteriovenous differences, Himwich *et al.* (12), Lennox *et al.* (13), Wortis *et al.* (14), and Courtice (15) have reported aver-

TABLE I

*Examples of Manometric and Analytical Results on Brain Suspensions*

The results are expressed per cc. of suspension containing 150 mg. of fresh weight of whole brain.

Substrate added	Time*	O <sub>2</sub> uptake	R.Q.	Reducing substances as glucose				Lactic acid	Pyruvic acid	Acetic acid
				Fermentable (glucose)	Hydrolyzed					
					Gross	Non-fermentable	Fermentable (total carbohydrate)			
		c. mm.		mg.	mg.	mg.	mg.	mg.	mg.	
Glucose, 1.5 mg. per cc.	Zero			1.27	2.16	0.71	1.45	0.36	0.006	0.03
	120 min.			0.39	1.16	0.62	0.54	0.61	0.011	0.07
	Change	474	0.92	-0.88			-0.91	+0.25	+0.005	+0.04
No addition	Zero			0.00	0.63	0.48	0.15	0.117	0.007	0.02
	120 min.			0.00	0.63	0.48	0.15	0.007	0.001	0.05
	Change	267	0.86	0.00			0.00	-0.110	-0.006	+0.03
No addition (insulinized rats)	Zero			0.00	0.72	0.62	0.10	0.037		0.04
	120 min.			0.01	0.68	0.55	0.13	0.024		0.06
	Change	159	0.80	+0.01			+0.03	-0.013		+0.02
Pyruvic acid, 2 mg. per cc.	Zero			0.011	0.74	0.54	0.20	0.193	1.75	0.02
	120 min.			0.017	0.77	0.56	0.21	0.354	0.70	0.11
	Change	472	1.20	+0.006			+0.01	+0.161	-1.05	+0.09
d-Lactic acid, 2 mg. per cc.	Zero			0.00	0.69	0.57	0.12	2.09	0.009	0.04
	120 min.			0.01	0.71	0.55	0.16	1.62	0.021	0.08
	Change	433	0.89	+0.01			+0.04	-0.47	+0.012	+0.04
Glucose + pyruvic acid	Zero			1.26	2.10	0.58	1.52	0.32	1.89	0.06
	120 min.			0.58	1.39	0.58	0.81	1.02	0.86	0.14
	Change	521	1.18	-0.68			-0.71	+0.70	-1.03	+0.08
Glucose + d-lactic acid	Zero			1.27	2.15	0.65	1.50	2.17	0.010	0.03
	120 min.			0.67	1.47	0.57	0.90	2.22	0.016	0.07
	Change	453	0.89	-0.60			-0.60	+0.05	+0.006	+0.04
Glycogen, 3 mg. per cc.	Zero			0.01	3.99	0.53	3.46	0.128		0.03
	120 min.			0.15	3.78	0.53	3.25	0.035		0.04
	Change	323	0.90	+0.14			-0.21	-0.093		+0.01

\* "Zero" samples were taken after 7 minutes shaking at 38°, about 15 minutes after mixing with the substrate. "120 min." samples were taken 120 minutes after zero time.

† Non-fermentable reducing substances were between 0.03 and 0.05 mg. (as glucose) per cc. Since they did not change significantly in amount during the experiments, they were not determined in all experiments.

age R.Q. values ranging from 0.95 to 1.0, with wide variations between individual determinations, for the respiration of the brains of animals and

TABLE II  
*Oxygen Uptake and CO<sub>2</sub> Evolution Accounted for by Changes in Substances Determined*  
 The results are expressed in c.mm. per gm. of whole fresh brain in 120 minutes.

	No. of experiments averaged	Substrate added	Gas exchange determined manometrically	p.q.	Gas exchange expected from changes in amount of				Gas exchange accounted for*		
					Glucose (total carbohydrate)*	Lactic acid	Pyruvic acid	Acetic acid	Sum of preceding columns	Per cent of manometrically determined	
										On average results	Individual experiments
Normal animals	5	Glucose	O <sub>2</sub>	-2975 +2720	0.91	-3980 (4155) +3980 (4155)	+1255 -1255	+10 -10	-2505 (2680) +2505 (2680)	84 (90) 92 (99)	82, 83, 91, 83, 80
	3	No addition	O <sub>2</sub>	-1760 +1530	0.87	+35 (-20)	-555 +555	-15 +15	-430 +430	24 28	29, 22, 23
	3	Pyruvate	O <sub>2</sub>	-3330 +4025	1.21	-10 (+35)	+650 -650	+15 +500	-3130 +3130	103 108	106, 99, 103
	4	d-Lactate	O <sub>2</sub>	-2970 +2610	0.88	+75 (+15)	-2400 +2400	+15 -55	-2165 +2155	73 83	76, 73, 67, 73
	4	Glucose + pyruvate	O <sub>2</sub>	-3245 +3835	1.18	-2990 (3140) +2990 (3140)	+3220 -3220	+120 +430	-3460 (3610) +4295 (4445)	107 (111) 112 (116)	103, 129, 90, 110
Insulinized animals	4	Glucose + d-lactate	O <sub>2</sub>	-2970 +2635	0.89	-2765 (2770) +2765 (2770)	+235 -235	+25 -30	-2305 (2310) +2300 (2305)	78 (78) 87 (87)	81, 73, 84, 70
	2	Glucose	O <sub>2</sub>	-3200 +2975	0.93	-4680 (4780) +4680 (4780)	+1825 -1825	0 +170	-2685 (2785) +2685 (2785)	84 (87) 90 (94)	81, 87
	1	No addition	O <sub>2</sub>	-1060 +850	0.80	+60 (+160)	-65 +65	+90 -90	±0 -2855	0 92	89, 96
	2	d-Lactate	O <sub>2</sub>	-3095 +2725	0.88	+10 (+200)	-3120 +3120	+45 -55	+220 -220	104	
			CO <sub>2</sub>								

\* Figures in parentheses are calculated on the basis of total carbohydrate change, others from glucose change. For experiments without added glucose, the slight and questionable changes found in glucose or total carbohydrate are not included in the sum.

normal human subjects *in vivo*. It was found that combustion of the glucose which disappeared from the blood during circulation through the brain, provided the level of blood sugar had not recently been greatly changed, could account approximately for the entire oxygen usage. With slices of rat brain cortex, Dickens and Simer (16) found r.q. values averaging 0.99 in phosphate-Ringer's medium, 0.98 in bicarbonate-Ringer. Elliott and Baker (17) found r.q. values averaging 0.86, but later Elliott *et al.* (18) and Baker *et al.* (19), observing certain precautions, found values averaging 0.94 in bicarbonate-Ringer.<sup>3</sup> With the whole brain suspensions used in the present work, the r.q. in the presence of glucose has varied between 0.90 and 0.94. The r.q. generally found *in vitro* is thus a little lower than seems to be the true value *in vivo*.

The averages of figures given by Baker *et al.* (19) indicated that complete combustion of the glucose disappearing from the medium, after making allowance for the unconsumed lactic acid formed from some of the glucose, could account for about 76 per cent of the oxygen uptake with rat cortex slices, 84 per cent with cat cortex slices. Results shown in Table II, obtained by a more accurate experimental procedure, confirm the conclusion that glucose metabolism can account for most of the respiration of brain tissue in glucose-containing medium. Slightly more total carbohydrate appeared to be used than glucose. The difference may be due to the fact that hexose phosphates would be more completely included in reducing values determined after hydrolysis.

If we assumed that all the carbohydrate not otherwise accounted for was completely oxidized, causing  $\text{CO}_2$  evolution and  $\text{O}_2$  absorption in equal amounts, it would appear that glucose (or total carbohydrate) metabolism could account for 92 (or 99) per cent of the respiratory  $\text{CO}_2$  but only 84 (or 90) per cent of the oxygen uptake. This might suggest that some other

<sup>3</sup> Dickens (20) maintained that the low figures of Elliott and Baker were contrary to the results of other workers. However, none of the references he cited as giving an r.q. of 1.0 seems valid. For instance, Meyerhof and Lohman (21) measured the "apparent r.q." in which  $\text{CO}_2$  liberated by acid is included with the respiratory  $\text{CO}_2$ . Papers by Himwich *et al.* were quoted, but Dr. Himwich informed us that he later obtained relatively low r.q. values, and such values are published by Himwich *et al.* (22, 19). Ashford and Holmes (23) mention an r.q. close to unity in text and summary but the mean figure appearing in their table is 0.91. The only figures given by Loebel (24) are for brain in the presence of fructose. Gerard and Schachter (25), also quoted, give no measurement of r.q. at all. A paper by Gayda was quoted but this was not available to us nor apparently to Dickens, since it was quoted at second-hand. The remainder of Dickens' criticisms could be similarly answered. However, Elliott *et al.* (18) noted that brain slices show strong aerobic glycolysis for a short time. This can cause  $\text{CO}_2$  liberation from the medium before the manometer taps are closed, and, unless this is carefully controlled, either high or low r.q. values may be obtained by the usual methods.

substances in the tissue were oxidized with very little  $\text{CO}_2$  evolution. More probably oxidation of the glucose was not quite complete; so that less than the calculated  $\text{CO}_2$  and oxygen exchanges should be ascribed to glucose oxidation and more than 16 (or 10) per cent of the oxygen uptake must be due to the combustion of non-carbohydrate material. Perhaps under normal conditions *in vivo* glucose oxidation is more complete and less non-carbohydrate oxidation occurs.

When glucose was added to a suspension, there was a rapid accumulation of lactate during the equilibration period followed by a slower increase in lactate concentration during the experiment. Similar observations were made with slices of cortex by Elliott *et al.* (18). This aerobic lactic acid accumulation may be a non-physiological phenomenon occurring only *in vitro*.

In the presence of glucose, brains from insulin-treated rats showed no significant differences from normal brains. The respiration was somewhat greater than the average but within the normal range.

In all experiments in the presence of glucose the rate of respiration was well maintained (Mg present, see (1)). The oxygen uptake in the fourth half hour was about 75 per cent of that in the first half hour.

*No Added Substrate*—In the absence of added substrate the respiration rate was initially slightly lower than in the presence of glucose and the rate fell off considerably more rapidly. Over a 2 hour period the total oxygen uptake was about 60 per cent of that found with glucose. Suspensions containing no added substrate always contained initially a small amount of lactic acid, about 0.13 mg. per cc. or 0.85 mg. per gm. of fresh tissue. This lactic acid was largely consumed during the experimental period, the greater part of it being used up in the 1st hour. Oxidation of this lactic acid accounted for about 24 per cent of the respiration over 2 hours. There was virtually no glucose present originally and the total carbohydrate showed no appreciable change; so that the remaining 76 per cent of the respiration must be due to the combustion of non-carbohydrate material. The rather low R.Q. of 0.87 reflects this.

The lactic acid found in the suspensions was presumably formed, during the preparation of the suspensions, by glycolysis of glucose present in the brain at death (26, 27). As was found by Holmes and Sherif (26) with mice, when the blood glucose of rats had been reduced by insulin treatment, only a trace of lactate was found in the brain suspension. The respiration of a suspension of the brains of insulinized rats without substrate addition was lower than that of normal brain suspensions; the R.Q. was 0.80 and lactate combustion could not account for more than 6 per cent of the respiration.

Holmes (28) observed that lactate oxidation could not account for the

respiration of normal or insulinized rabbit or mouse brain when no substrate had been added. He concluded that considerable non-carbohydrate oxidation occurred in brain and assumed that this would continue in the presence of glucose. The data here presented indicate that glucose suppresses much of the oxidation of non-carbohydrate material. With normal brains, when no substrate had been added, the oxygen consumed by non-carbohydrate material was equal to about 46 per cent (76 per cent of 60 per cent) of the total oxygen uptake observed in the presence of glucose. But only about 16 per cent of non-carbohydrate oxidation actually occurs in the presence of glucose. However, it seems probable that, under conditions of severe hypoglycemia, the brain *in vivo* may oxidize non-carbohydrate material, though such oxidations, at least during coma, are not adequate to support normal brain functions.

*Pyruvate*—In the presence of added pyruvate the respiration rate was somewhat greater than in the presence of glucose. The R.Q. averaged 1.21 and the oxygen uptake and CO<sub>2</sub> evolution were slightly less than could be accounted for by the pyruvate disappearing after allowance was made for the lactate and acetate formed. Apparently small amounts of other products of incomplete combustion were formed. Pyruvate thus seems to be even more active than glucose in suppressing all the non-carbohydrate oxidations which occur in the absence of added substrate.

Krebs and Johnson (29) and Elliott *et al.* (18) showed that, anaerobically, various sliced tissues including brain cause some dismutation of pyruvate whereby 2 molecules of the latter yield 1 molecule each of acetate, lactate, and CO<sub>2</sub>. Long (30) estimated that about 25 per cent of the pyruvic acid metabolized aerobically by pigeon brain mash was converted to acetic acid, 5 per cent by dismutation and 20 per cent by direct oxidation. Long did not actually determine the total pyruvic acid utilized. With rat brain we found that on the average only about 14 per cent of the pyruvic acid metabolized was converted to acetic acid. Since nearly an equivalent amount, about 12 per cent of the pyruvic acid, was reduced to lactic acid, dismutation could account for almost all the acetic acid formed and there was very little formed by direct oxidation. However, there is probably no fundamental difference between oxidation and dismutation; it is probably merely a question of whether other pyruvate molecules or other substances accept the hydrogen made available in the oxidation of pyruvate to acetate and CO<sub>2</sub>.

It should be noted that the amount of lactate formed from pyruvate was less than the amount which accumulated by aerobic glycolysis from glucose. The equilibrium between pyruvate + reduced DPN<sup>4</sup> and lac-

<sup>4</sup> Diphosphopyridine nucleotide.



tate  $\div$  DPN is well known to tend strongly toward reduction of pyruvate to lactate. But it seems that, in the absence of metabolites formed from glucose, other reducing systems are not sufficiently active to keep the DPN reduced; so that there is less tendency for pyruvate to be reduced and more tendency for it to undergo oxidative metabolism.

Previous workers (see *e.g.* (4)) have shown that acetoacetate is formed from pyruvate by liver tissue. No acetoacetate formation by brain tissue was detected by the aniline citrate method (31).

When pyruvate and glucose were added together, the oxygen uptake was about the same as with pyruvate alone and greater<sup>5</sup> than with glucose alone. When large amounts of two substrates were present, less accuracy and wider variations were to be expected in the balance sheet. But the main observations were consistent and far beyond experimental error. Both substances disappeared but the consumption of pyruvate was considerably greater than that of glucose. Pyruvate seems clearly to be able to compete with glucose as a substrate for brain respiration. This would be expected if pyruvate were an intermediary product of glucose metabolism. The lactate production was greater than the sum of the amounts found with glucose or pyruvate alone. Stimulation of glycolysis by pyruvic acid is well known.

Traces of material estimated as pyruvate were always found in experiments with added glucose or lactate or no added substrate, but changes in the amount were not large enough appreciably to affect the balance sheets for those experiments.

*Lactate*—In the presence of added lactate, the respiration rate was initially somewhat greater than with glucose but fell off rather more rapidly; so that the total oxygen uptake over 2 hours was about the same with glucose or lactate. Lactate combustion accounted for only about 73 per cent of the total respiration and the r.q. of 0.88 was slightly lower than was found with glucose. Apparently lactic acid cannot suppress the oxidation of non-carbohydrate material as efficiently as does glucose. Ashford and Holmes (23) found with chopped rabbit brain and high lactate concentrations, 0.03 M or higher, presumably *dl*-lactate, that about 35 per cent more lactic acid disappeared than could be accounted for. This was especially marked if the tissue had been depleted of its own substrates by various methods such as previous insulinization of the animal. Our results, with 0.022 M natural *d*-lactate on normal or insulinized rat

<sup>5</sup> With slices of cortex, Elliott *et al.* (18) found that the respiration in the presence of glucose was somewhat decreased by the addition of pyruvate. This and the fact that isotonic sucrose is not as effective as NaCl in maintaining the respiration rate of slices (1) are the only qualitative differences in behavior which we have observed between slices and isotonic suspensions of brain.

brains, do not confirm this finding. With brains from insulinized animals the lactic acid utilization could account for a greater proportion of the oxygen uptake than with normal brains, about 92 per cent. (The  $\text{CO}_2$  evolution was possibly slightly more than 100 per cent accounted for. Probably traces of unconsumed intermediate oxidation products accumulate.) Similar results were reported by Dixon (32), working with rabbit cortex slices. During the period of insulin hypoglycemia much of the non-carbohydrate substrate of brain respiration is presumably consumed and the remainder competes less effectively with added lactate for the respiratory mechanisms.

When lactate and glucose were added together, the oxygen uptake found was about the same as with either alone. The respiration was largely accounted for by glucose consumption and lactate did not disappear but rather its amount still increased slightly. (In one experiment, when the respiration was unusually low as a result of damage due to a very tight homogenizer, there was some disappearance of lactate.) Similar results were found by Elliott *et al.* (18) with slices of cortex. A possible explanation of these results could be that lactate is formed from glucose and then oxidized almost as fast as it is formed. But it is now commonly believed that lactate is not a direct intermediate in carbohydrate oxidation and is only produced from the actual intermediate, pyruvate, by reduction when the pyruvate is not immediately oxidized. It seems that lactate cannot compete with glucose as a substrate of brain respiration.

**Total Carbohydrate**—Elliott and Libet (33) reported that glycogen was oxidized by brain suspensions, the mechanism being different from that involved in glucose or pyruvate oxidation. This has now been shown to be incorrect (34). The substance causing the increased respiration was an unknown impurity present in the glycogen preparations tested and also found in other materials. Glycogen, free of this impurity,<sup>6</sup> had practically no immediate effect on the rate of respiration of brain suspensions but the rate fell off less rapidly than when no substrate was added. This was almost certainly due to the slow production of glucose from the added glycogen. It will be seen in Table I that glucose was formed, while the total carbohydrate decreased sufficiently to account for part of the respiration.

The total fermentable reducing material after hydrolysis exceeded the amount of glucose in the suspensions on the average by about 0.9 mg. (as glucose) per gm. of tissue when no glucose was present, about 1.2 mg. per gm. when glucose was present. This excess would consist of hexose phosphates and other hydrolyzable sugar derivatives as well as glycogen. Kerr and Ghantus (35) found about 1 mg. of glycogen per gm. of dog or rabbit

<sup>6</sup> Glycogen, free of N and P, kindly provided by Dr. M. Somogyi, was used.

brain frozen *in situ* and showed that the amount decreased markedly on treatment with insulin. During the preparation of our suspensions the glycogen may have largely disappeared; changes in the amount of carbohydrate other than glucose during the experiments, whether substrate was added or not, were usually slight, probably within the experimental error. Appreciable utilization of non-glucose carbohydrate thus did not occur, but the experiments with added glycogen mentioned above and the observations of Kerr and Ghantus indicate that glycogen can be utilized to some extent by brain when glucose is lacking. There was no sign of appreciable synthesis of carbohydrate from lactate or pyruvate except possibly with insulinized brains. Benoy and Elliott (36) found no carbohydrate synthesis from lactate or pyruvate with slices of rat cerebral cortex and Ashford and Holmes (23) found none with lactate and chopped rabbit brain.

The amount of non-fermentable reducing substances found after hydrolysis was quite high, on the average equivalent to about 4.2 mg. of glucose per gm. of fresh tissue. There was usually a slight decrease during the experiments, the final average being about 3.9 mg., but since it is not known what substance was concerned in the decrease, no account has been taken of it in the balance sheets.

*Acetic Acid*—In the presence of added sodium acetate, 0.022 M, the oxygen uptake rate and R.Q. were exactly the same as with no added substrate and there was no sign that acetate was utilized. (Found, 1.38 mg. of acetic acid per cc. of suspension initially, 1.47 mg. after 2 hours respiration.) Acetate has no effect on brain cortex slices (18). It appears, therefore, that free acetic acid cannot be a normal brain metabolite. But appreciable amounts of acetic acid were found when added pyruvate was metabolized and definite traces of a substance estimated as acetic acid were always present initially and increased in amount during the metabolism of other substrates. The acetic acid found might exist in the tissue as acetyl phosphate or some other labile compound which is hydrolyzed during the determination, or acetic acid itself might be a product formed as a result of unfavorable conditions of *in vitro* work.

*Effects of Concentration of Glucose, Lactate, and Pyruvate*—In Fig. 1, the effects of various concentrations of added glucose, lactate, and pyruvate on the initial rate of oxygen uptake are shown. In order that the concentrations of the smaller amounts of added substrates should not be too rapidly changed by utilization, smaller amounts of tissue than usual were used in the manometer flasks and the substrate solutions were added from side bulbs after 5 minutes equilibration and only 2 minutes before the taps were closed.

It will be seen that the maximum initial rate of respiration in the presence

of glucose was reached with 10 mg. per cent ( $5.6 \times 10^{-4}$  M) or less of glucose, and further moderate increase in concentration had no additional effect. Bernheim and Bernheim (37) obtained half maximal effects on washed brain suspensions with  $1.4 \times 10^{-4}$  M glucose. In some experiments, six out of eleven, increasing the glucose concentration up to 200 mg. per cent caused some decrease in initial rate (see Fig. 1), while in five experiments the rate was constant up to this concentration. (In one experiment it was constant up to 400 mg. per cent.) The reasons for the decrease at high glucose concentration and for the variability of the effect are not understood.

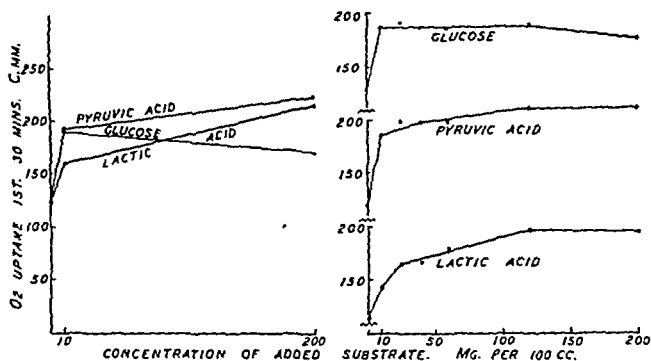


FIG. 1. Effects of varying concentrations of glucose, lactate, and pyruvate on the initial oxygen uptake of isotonic brain suspensions, 200 mg. of whole brain. The three curves on the right are not directly comparable with each other, since they were obtained with different, but similarly prepared, suspensions. Concentrations are given in terms of pyruvic and *d*-lactic acids, though the sodium salts were used.

With pyruvic acid at 10 mg. per cent,  $11 \times 10^{-4}$  M, the rate was about equal to that with the glucose. The rate increased a little as the pyruvate concentration was raised up to about 100 mg. per cent. Added *d*-lactic acid, 10 mg. per cent, affected the respiration rate considerably less than did glucose or pyruvic acid. The initial rate increased more gradually with increasing lactate concentration, reached the glucose level at about 50 mg. per cent, and surpassed it at higher concentrations.

It will be noted that in the work reported in the preceding sections approximately optimum concentrations of substrates were used.

#### DISCUSSION

The ready utilization of lactate and pyruvate by brain tissue *in vitro* is well known. Yet the work of a number of authors has indicated that these

substances cannot replace glucose as substrate for brain metabolism *in vivo*. Wortis *et al.* (38, 39) found that, while intravenous injection of as little as 4 gm. of glucose would rouse patients from coma due to insulin, injections of 20 gm. of sodium *dl*-lactate, or 4 to 8 gm. of sodium pyruvate, did not in general rouse the patients at all. While glucose doubled the oxygen uptake rate of the brain, lactate usually caused less increase and the effect of pyruvate was small. Elliott *et al.* (5) found no correlation between the level of endogenous blood lactate and pyruvate and the state of consciousness of patients in insulin hypoglycemia. Mann and Magath (40) and Wortis *et al.* (39) were unable to relieve the convulsions of hepatectomized animals with sodium lactate, though glucose injections did so readily. With abdominally eviscerated hepatectomized rabbits, Maddock *et al.* (41) found that the cortical electrical potentials could be restored by injections of glucose but not by a number of other substances, including pyruvate.

In the preceding sections we have demonstrated three differences between the behavior of glucose and lactate as substrates for brain metabolism. (1) Lactate is not as efficient as glucose in displacing non-carbohydrate metabolism. (2) Glucose is utilized to the exclusion of lactate when glucose and lactate are both present, indicating that glucose is a "preferred" substrate. (3) At low concentrations, lactate stimulates brain respiration less than does glucose. These observations may help to explain the inability of administered lactate to replace glucose for normal brain function *in vivo* but do not solve the problem. Though lactate oxidation accounts for a lower proportion of the respiration than does glucose with brains from normal animals, the proportion is at least as high as for glucose with brains from insulinized animals. The blood lactic acid level produced by Wortis *et al.* (38) in insulinized patients reached an average of 75 mg. per cent, though the concentration of natural *d*-lactate must have been considerably lower since the *dl* salt was administered. But very high endogenous blood lactate levels, 100 mg. per cent, occurred in the hepatectomized dogs of Wortis *et al.* without protecting the animals from convulsions. It remains to be determined whether high blood concentrations of natural lactic acid can increase the oxygen uptake of the brain *in vivo* in insulinized subjects to the same level as glucose can. If this does not occur, it is possible that permeability factors limit accessibility of lactate to the brain cells. If it is found that high blood lactate can produce the same oxygen uptake as glucose does and still not affect convulsions, consciousness, or electrical potentials, it will be necessary to assume that certain particular reactions in the metabolism of glucose are necessary for normal brain functions.

The *in vitro* studies indicate that, unlike lactate, pyruvate is quite as efficient a substrate for brain respiration as glucose. Pyruvate seems to

displace all non-carbohydrate oxidations, it is used more rapidly than glucose when both are present, and at low concentrations the oxygen uptake rate is equal to that with glucose. Possibly adequate blood concentrations of pyruvate would stimulate normal brain function *in vivo* as well as glucose does. But it would be difficult to maintain a sufficiently high blood pyruvate concentration for long enough to study its effects, since pyruvate is reduced to lactate or otherwise metabolized very rapidly in the rest of the body. Flock *et al.* (42) found that injections into normal dogs of 0.25, 0.5, and 1.0 gm. of pyruvic acid per kilo per hour raised the blood pyruvate to about 6, 20, and 72 mg. per cent respectively. Injection of 1 gm. of pyruvic acid per kilo would be equivalent to 87 gm. of sodium pyruvate for a 70 kilo man. Again, of course, permeability factors may enter or the metabolism of pyruvate may not include particular reactions necessary for proper brain function.

#### SUMMARY

1. Adaptations of known methods for the determination of glucose and total carbohydrate and lactic, pyruvic, and acetic acids in isotonic rat brain suspensions have been worked out. Corrections are shown to be necessary in most cases when pyruvate is present.

2. In the absence of added substrate, oxidation of non-carbohydrate materials accounts for most of the respiration of isotonic brain suspensions.

3. In the presence of glucose, the oxidation of non-carbohydrate materials by brain suspensions is largely suppressed and nearly all the respiration takes place at the expense of glucose. The maximum rate of respiration occurs in the presence of 10 mg. per cent of glucose.

4. In the presence of pyruvate, the respiration can be accounted for completely by pyruvate utilization. About 14 per cent of the pyruvate utilized is converted to acetate and an approximately equivalent amount of lactate is formed. Respiration rates equal to those with glucose are obtained in the presence of 10 mg. per cent of pyruvic acid. When pyruvate and glucose are both present, more pyruvate than glucose disappears.

5. With suspensions from normal brains, added lactate is not as efficient as glucose or pyruvate in displacing non-carbohydrate oxidations, but with brains from insulinized animals oxidation of added lactate can account for approximately the entire respiration. Higher concentrations of lactate than of glucose or pyruvate are required to produce the maximum respiration rate. When lactate and glucose are both present, glucose only is utilized, while the lactate concentration is usually not decreased.

6. No appreciable utilization of carbohydrate other than glucose could be detected in the presence or absence of the added substrates and there was no significant sign of synthesis of carbohydrate from lactate or pyruvate.

7. Acetic acid has no effect on the oxygen uptake or n.q. and is not utilized by brain suspensions.

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## LETTERS TO THE EDITORS

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### pH CHANGE AS A MEASURE OF GROWTH OF LACTOBACILLUS CASEI IN VITAMIN ASSAYS

Sirs:

In our experience with the pantothenic acid assay procedure,<sup>1</sup> we have had occasion to make certain modifications as follows:

Direct pH determination after the incubation period has been substituted for titration or turbidity measurements as a measure of growth of the organism. A spread of about 2 pH units is obtained with a working range covering 0.02 to 0.10  $\gamma$  of pantothenic acid per tube. Frequently, valid assays are obtained with even less than 0.01  $\gamma$  per tube or as much as 0.15  $\gamma$ . With the Beckman pH meter, model G, it is possible to make pH measurements on 120 samples per hour. One washing of the electrode and container between readings is adequate.

Assays of the blood, tissue, feces, and urine of dogs can be made after 24 hours incubation instead of the customary longer periods with no decrease in accuracy. Simultaneous assays of tissues, feces, and urine with both 24 and 72 hour incubation periods as well as both titration and pH methods of measuring growth yielded values which checked within 10 per cent. However, the titration curve was not as satisfactory as the pH curve after 24 hours incubation. Quantitative recovery of added pantothenic acid was observed in all cases. Dog blood assays with the pH method (2 cc. of untreated dog blood added to the medium for 100 tubes) agreed at 24 and 72 hours when 0.1 to 0.2 cc. of laked blood was added to each tube, with or without added pantothenic acid (0.02  $\gamma$  per tube).

Growth is inhibited in all tubes simultaneously by chilling in the refrigerator. Each basket of forty tubes is then warmed to 26–27° in a water bath before the pH determinations are made.

In order to insure a dependable medium for uninterrupted work, the practice of preparing fresh medium for each assay has been abandoned. Ample amounts of the various ingredients are made up and, after a check assay, a series of bottles of undiluted stock medium is prepared (without dextrose), plugged, autoclaved, and stored in the cold until needed. This medium keeps at least 2 to 3 months. The cystine is made up in a concentration of 50 mg. per cc. as the hydrochloride which does not precipitate on standing. The medium is filtered before use and is adjusted to pH 6.9 to 7.0 (autoclaving lowers this subsequently to 6.6 to 6.7).

The foregoing procedure is also used for riboflavin assays with minor alterations. In fact, the undiluted stock medium can be made up and

<sup>1</sup> Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **135**, 213 (1940).

stored without the yeast extract and riboflavin (or calcium pantothenate), so that it may be used for either assay merely by addition of the appropriate yeast extract, vitamin supplement, and dextrose.

Advantages of this procedure are greater convenience, speed, objectivity, and wider applicability.

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Received for publication, September 21, 1942

# REVERSAL BY PHOSPHATIDES OF THE ANTIMICROBIAL ACTION OF A CRYSTALLINE PROTEIN FROM WHEAT

Sirs:

Stuart and Harris<sup>1</sup> have shown the antimicrobial and especially the yeast-inhibiting action of a protein isolated from wheat.<sup>2</sup> Since the toxic effect of antibiotin from egg white<sup>3,4</sup> and of antithiamine from carp<sup>5</sup> is the result of inactivation of these vitamins in the medium, and apparently not of the direct poisonous action on the cell,<sup>4</sup> it seemed possible that the antimicrobial action of the protein from wheat might be due to its anti-vitamin action in combining with a vitamin in the medium. This idea was examined with a quantity of the crystalline protein generously supplied by Dr. A. K. Balls. While no direct antivitamin action was found, the following facts were established.

*Effect of Phosphatides on Growth (Acid Production) of Lactobacillus casei in Presence of 20  $\gamma$  per Cc. of Protein*

Additions	Amount	0.1 N acid produced
	$\gamma$ per cc.	cc. per 10 cc. culture
None .....		2.2
Lipositol.....	100	13.4
" .....	10	1.3
Lecithin.....	100	11.8
" .....	10	4.2
Phosphatidyl serine.....	100	12.7
" " .....	10	4.6

Preliminary experiments showed that, while growth of yeast in a medium of salts and glucose was inhibited by the protein, it was not inhibited in the richer medium of Woolley.<sup>6</sup> Thus while 20  $\gamma$  of protein per cc. prevented the growth of yeast in the former, 50  $\gamma$  did not inhibit it in the latter. Evidently a water-soluble material in the latter medium was inhibiting the action of the protein. It was also found that *Lactobacillus casei* grown on the medium of Pennington *et al.*<sup>7</sup> plus pantothenic acid was inhibited by smaller quantities of protein than was yeast. Since *Lactobacillus casei* was

<sup>1</sup> Stuart, L. S., and Harris, T. H., *Cereal Chem.*, **19**, 288 (1942).

<sup>2</sup> Balls, A. K., Hale, W. S., and Harris, T. H., *Cereal Chem.*, **19**, 279 (1942).

<sup>3</sup> Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **136**, 801 (1940).

<sup>4</sup> Woolley, D. W., and Longworth, L. G., *J. Biol. Chem.*, **142**, 285 (1942).

<sup>5</sup> Woolley, D. W., *J. Biol. Chem.*, **141**, 997 (1941).

<sup>6</sup> Woolley, D. W., *J. Biol. Chem.*, **140**, 453 (1941).

<sup>7</sup> Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **135**, 213 (1940).

not inhibited when grown in peptone medium, it was concluded that the inhibition here too was antagonized by some material in peptone.

Since exploratory experiments indicated some action of inositol in antagonizing the inhibition of yeast in a glucose-salts medium, the effect of lipositol, the inositol-containing phosphatide<sup>8</sup> studied in this laboratory, was tested. This compound completely reversed the inhibition of growth of yeast or *Lactobacillus casei* by the protein. In addition, lecithin and phosphatidyl serine<sup>9</sup> (both free of inositol) were almost equally effective. (Compare results with gramicidin<sup>10</sup> and detergents.<sup>11</sup>)

When growth of *Lactobacillus casei* was prevented by the protein, the cells of the inoculum did not die, for, when the phosphatide was added after 24 hours of incubation, growth and acid production ensued.

Thus it seems that the antimicrobial action of the crystalline protein may be prevented by constituents of the medium. Since several phosphatides were almost equally effective and since the protein occurs in wheat in combination with phosphatide,<sup>2</sup> the reversal of its antimicrobial action may be due to formation of a lipoprotein. This complex may not penetrate the cell.

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<sup>8</sup> Folch, J., and Woolley, D. W., *J. Biol. Chem.*, **142**, 963 (1942).

<sup>9</sup> Folch, J., *J. Biol. Chem.*, **139**, 973 (1941).

<sup>10</sup> Dubos, R. J., and Hotchkiss, R. D., *Tr. Coll. Physn. Philadelphia*, **10**, 11 (1942).

<sup>11</sup> Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, **74**, 621 (1941).

## ISOMERIZATION OF CHLOROPHYLLS A AND B

Sirs:

By chromatographic adsorption of plant extracts, we have found that chlorophylls *a* and *b* are accompanied by smaller quantities of two other green pigments. One of these, *chlorophyll a'*, is blue-green like chlorophyll *a*. The other, *chlorophyll b'*, is yellow-green like chlorophyll *b*. The spectral absorption properties of chlorophylls *a* and *a'* are remarkably similar, as are also those of chlorophylls *b* and *b'*. Consequently it is difficult to detect the chlorophylls *a'* and *b'* in leaf extracts by means of spectral absorption measurements.

Higher plants and green algae extracted at room temperature or above yield these two additional chlorophylls. However, only traces of the new pigments are obtained from plant extracts prepared quickly at low temperature ( $\sim 80^\circ$ ). Plants which do not contain chlorophyll *b*, as for example diatoms and brown algae,<sup>1</sup> do not yield chlorophyll *b'*.

Chlorophylls *a* and *a'* are interconvertible, as are also chlorophylls *b* and *b'*. At room temperature, chlorophylls, in solution or on the adsorption columns, are interconverted very slowly. At  $95\text{--}100^\circ$  in *n*-propanol this interconversion takes place rapidly, yielding an equilibrium mixture which contains roughly one-fifth of the new isomer and four-fifths of the common form. Exposure of plant material to a temperature of  $100^\circ$  also causes interconversion of the green pigments. This ready interconversion may make it very difficult to determine whether or not chlorophylls *a'* and *b'* are natural plant constituents.

Different pheophytins are obtained from the two *a* isomers. This indicates that the difference between chlorophylls *a* and *a'* involves the organic portion of the molecule rather than the attachment of the magnesium. With alcoholic KOH, both chlorophylls *a* and *a'* yield spectroscopically similar (or identical) pheopurpurins that are not extractable from ether with 12 per cent hydrochloric acid. This indicates that *chlorophyll a'* probably does not correspond to the hypothetical chlorophyll *a*<sub>2</sub> of Conant and Dietz.<sup>2</sup>

Chlorophylls *a'* and *b'* may be prepared easily by the following procedure. Green leaf tissue (20 gm.) is scalded in boiling water for 1 minute. It is cooled and extracted with acetone or with methanol. Pigments in the extract are transferred to petroleum ether by the addition of this solvent and a little water. After removal of the aqueous layer, the petroleum ether is extracted once or twice with 80 per cent methanol and thrice with

<sup>1</sup> Strain, H. H., and Manning, W. M., *J. Biol. Chem.*, **144**, 625 (1942).

<sup>2</sup> Conant, J. B., and Dietz, E. M., *J. Am. Chem. Soc.*, **55**, 839 (1933).

water. The green petroleum ether solution is filtered through an adsorption column (6.7 by 18 cm.) of dry powdered sugar,<sup>1, 3</sup> whereupon the green pigments are strongly adsorbed. They are then separated into discrete bands by washing the column with petroleum ether containing 0.5 per cent *n*-propanol and 0.5 per cent dimethylaniline<sup>1</sup> (carefully purified). Chlorophyll *a'*, which forms the lowest green band, separates slowly from the similar green chlorophyll *a* band. Chlorophyll *b'* forms a yellow-green band between that of chlorophyll *a* and the topmost yellow-green band of chlorophyll *b*.

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Received for publication, October 19, 1942

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<sup>3</sup> Strain, H. H., Chromatographic adsorption analysis, New York, 42 (1942).

# *p*-AMINOBENZOIC ACID, A GROWTH FACTOR FOR ACETOBACTER SUBOXYDANS

Sirs:

While *p*-aminobenzoic acid has been demonstrated to be important in the metabolism of higher plants, animals, and many bacteria, only *Clostridium acetobutylicum*<sup>1</sup> and a *Neurospora* mutant<sup>2</sup> have been reported to require the addition of this compound to the culture medium. It would be desirable, especially for comparative purposes, to have available other organisms requiring *p*-aminobenzoic acid for growth. *Acetobacter suboxydans* has now been added to this list. This organism is of particular interest because of its ability to oxidize polyhydric alcohols to hydroxy acids and ketones.

Details concerning the media employed and the handling of the cultures are given in another communication.<sup>3</sup> The culture used is a strain received from the American Type Culture Collection as No. 621.

Supplements to basal medium	Evelyn readings at 660 mμ
Uninoculated basal.....	100
<i>p</i> -Aminobenzoic acid, 0.0 γ per 10 cc.....	94
“ “ 0.003 γ “ 10 “ .....	93
“ “ 0.010 γ “ 10 “ .....	76
“ “ 0.050 γ “ 10 “ .....	21
Difco yeast extract, 50 mg. per 10 cc.....	19

The basal is a glycerol-hydrolyzed casein-salts medium to which the other vitamins required by this organism have been added; *viz.*, 10 γ of calcium pantothenate and 10 γ of nicotinic acid per 10 cc. It differs from the basal medium of Underkofler *et al.*<sup>3</sup> only in that the hydrolyzed casein has been treated with norit and extracted at pH 1.5 with ether to remove *p*-aminobenzoic acid. After 48 hours incubation the surface growth is homogenized and the turbidity is measured in an Evelyn photoelectric colorimeter at 660 mμ. The results are illustrated in the accompanying table. Maximum growth is obtained on addition of 0.05 γ of *p*-amino-

<sup>1</sup> Rubbo, S. D., and Gillespie, J. M., *Nature*, **146**, 838 (1940). Lampen, J. O., and Peterson, W. H., *J. Am. Chem. Soc.*, **63**, 2283 (1941).

<sup>2</sup> Tatum, E. L., and Beadle, G. W., *Proc. Nat. Acad. Sc.*, **28**, 234 (1942).

<sup>3</sup> Underkofler, L. A., Bantz, A. C., and Peterson, W. H., *J. Bact.*, in press.



benzoic acid per 10 cc. These results suggest the possibility of using this organism for *p*-aminobenzoic acid assay.

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# COLORIMETRIC DETERMINATION OF SERINE

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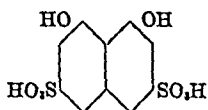
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Nicolet and Shinn (1, 2) were the first to show that serine on oxidation with periodate yields formaldehyde quantitatively. The periodate reacts with serine according to the equation,  $\text{HCH(OH)·CH(NH}_2\text{)·COOH} + \text{NaIO}_4 = \text{HCHO} + \text{NH}_3 + \text{CHO·COOH} + \text{NaIO}_3$ .

The formaldehyde was estimated by weighing the precipitate formed with dimedon according to Vorländer's (3) procedure. Threonine on oxidation with periodic acid yields acetaldehyde quantitatively, which also precipitates with dimedon. In order to determine serine in amino acid mixtures the acetaldehyde was removed by aeration and the formaldehyde precipitated in the reaction mixture. Analysis of aliquots containing 10 to 20 mg. of serine have so far been reported. Martin and Synge (4) also report reactions of periodate with hydroxyamino acids. Van Slyke *et al.* (5) have determined the hydroxylysine content of protein hydrolysates by estimating the  $\text{NH}_3$  liberated in the periodate reaction.

Our interest in this procedure arose from the necessity of determining serine accurately in amounts not exceeding 1 to 5 mg. in amino acid mixtures which originally contained a preponderance of cysteine. The possible interference of this amino acid in the serine determination has evidently not previously been considered.

We have found that formaldehyde can be quantitatively distilled from the reaction mixture without decomposition of periodic acid in the presence of adequate amounts of arsenite. The formaldehyde thus obtained is estimated colorimetrically with the aid of Eegriwe's reagent (6) 1,8-dihydroxynaphthalene-3,6-disulfonic acid (chromotropic acid), the error not



1,8-Dihydroxynaphthalene-3,6-disulfonic acid

exceeding 1 to 2 per cent. This reagent is specific for formaldehyde and produces no color with acetaldehyde or higher homologues. 50 to 100  $\gamma$  of formaldehyde in a volume of 50 cc. produced adequate color for estimation in a Duboscq colorimeter. If the reagent is purified and the color

developed according to the directions given below, color production is proportional to the concentration (within 2.0 per cent) when the unknown differs from the standard by not more than 50 per cent. The procedure has the additional advantage in that it is completed in a few minutes. Dimedon precipitation, on the other hand, is not completed in less than 48 to 72 hours.

*Reagents—*

Potassium arsenite, 25 per cent. 25 gm. are dissolved in water and made up to 100 cc.

Periodic acid, 0.5 M. 11.4 gm. are dissolved in water and made up to 100 cc. and filtered if not clear.

Methyl red; saturated aqueous solution in 0.05 N HCl.

Formaldehyde stock standard. 100 gm. of paraformaldehyde in 100 cc. of water are hydrolyzed with 20 cc. of 2 N  $\text{H}_2\text{SO}_4$  by heating at  $90^\circ$  until no precipitate of paraformaldehyde remains. It is then steam-distilled according to the procedure of Weinberger (7). The distillate is returned to the original flask and redistilled after addition of 10 cc. of 2 N  $\text{H}_2\text{SO}_4$ . The stock formaldehyde solution thus obtained should be approximately 6 M. If 1 cc. of 2 N  $\text{H}_2\text{SO}_4$  per liter is added to the stock solution, no perceptible change in the concentration of the formaldehyde takes place over a period of a month. It is standardized essentially according to the procedure of Romijn (8) as follows:

An aliquot equivalent to 0.2 to 0.3 cc. of the stock solution is added to 20 cc. of 0.12 N KCN. To this are added 25 cc. of standard 0.100 N  $\text{AgNO}_3$  and 3 cc. of concentrated  $\text{HNO}_3$ . The solution is diluted to 100 cc. in a volumetric flask, filtered, and 75 cc. of the filtrate are titrated with 0.1 N  $\text{NH}_4\text{SCN}$ , 5 cc. of saturated ferric ammonium sulfate being used to detect the end-point. The KCN solution is standardized by the same procedure except that no aldehyde is added.

Working standard. A portion of the stock standard is diluted with water to contain 15  $\gamma$  of formaldehyde per cc. This standard should be discarded if it is more than 24 hours old.

Chromotropic acid, 0.1 M. 0.9 gm. of the crystals is dissolved in 25 cc. of water in a small vessel which can be stoppered. Approximately 50 mg. of  $\text{SnCl}_2$  are added, and the solution shaken and centrifuged until clear. The solution keeps for at least 2 days if it is not exposed to the air except for withdrawal of samples.

Purification of chromotropic acid. 25 gm. of the impure, colored chromotropic acid are dissolved in 100 cc. of water in a flask on a steam bath. 2 gm. of  $\text{PbCO}_3$  are added. After solution has occurred,  $\text{H}_2\text{S}$  is bubbled through the solution until all the lead is precipitated. The flask is stoppered and the  $\text{PbS}$  allowed to settle. If the supernatant liquid is

not pale yellow, more  $\text{PbCO}_3$  and  $\text{H}_2\text{S}$  are added. The solution is filtered or centrifuged hot without contact with air. The flask is stoppered and the substance crystallized at  $4^\circ$ , filtered with suction, washed with alcohol and ether, and dried in a vacuum desiccator. The product should be nearly white.

### *Procedure*

A neutral solution containing 1 to 5 mg. of serine and 3 drops of methyl red is introduced into a 300 cc. Kjeldahl flask.<sup>1</sup> To this are added 4 cc. of 25 per cent of potassium arsenite and 2.5 to 2.8 cc. of 0.5 M periodic acid. The last 0.5 cc. of periodic acid is added drop by drop with continuous gentle shaking. Addition of periodic acid should be stopped when the mixture is acid to methyl red. A pinch of talc and enough water to make the final volume 70 cc. are now added. The Kjeldahl flask is placed in an upright position and connected to a vertical water condenser, the cooled surface of which is 25 to 35 cm. in length. Sufficient water to cover the end of the condenser tube (5 to 10 cc.) is placed in a receiver and the solution distilled over until about 5 cc. remain in the Kjeldahl flask. Several times during the distillation the contents of the flask are given a swirl to wash down the iodate, which splatters and becomes dried on the sides of the flask. This precaution should be observed particularly near the end of the distillation. Distillation is complete after 10 to 12 minutes of vigorous boiling. The final volume of the distillate is made to 100 cc.

*Colorimetric Estimation*—5 cc. of standard formaldehyde solution (containing 15  $\gamma$  per cc.) are pipetted into a 1  $\times$  8 inch test-tube graduated at 50 cc. An amount of the distillate containing from 50 to 100  $\gamma$  of formaldehyde is added to a similar tube. To each tube is added 0.5 cc. of 0.1 M chromotropic acid and enough water to make the final volume 17 cc. The tubes are cooled in an ice bath and 10 cc. of concentrated sulfuric acid added to each during 40 to 45 seconds with gentle shaking. The tubes are kept in the bath until the solutions are again cooled to the temperature of the bath. They are then made to the mark by allowing concentrated sulfuric acid to flow down the center of the tubes. The temperature of the contents rises to about  $80^\circ$ . The tubes are now heated for 10 minutes

<sup>1</sup> Amounts of serine as small as 200  $\gamma$  would presumably be adequate for analysis if the distillation procedure was modified to recover the formaldehyde quantitatively in 17 cc. or less of distillate. The distillation procedure adopted permits analysis of serine in amounts which are likely to be frequently available. It has the advantage of simplicity and affords the opportunity of repeating the colorimetric estimation on more than one aliquot of the distillate. The colorimetric estimation can therefore be made with an optimum amount of formaldehyde, even though the serine content of the unknowns varies over a wide range.

in a boiling water bath, cooled to room temperature, and color comparison made in a colorimeter equipped with paraffined or all-glass cups, within an hour. Micrograms of formaldehyde  $\times 3.50$  = micrograms of serine.

### Results

*Proportionality*—Table I shows that the intensity of color produced by the reaction of formaldehyde with chromotropic acid is proportional to the concentration of the formaldehyde within 2.0 per cent, provided the unknown differs by not more than 50 per cent from a standard containing 1.4 to 1.5  $\gamma$  per cc. The deviation from proportionality may be 4 to 5 per cent if an unknown differs by 50 per cent from a standard containing only 0.7  $\gamma$  of formaldehyde per cc. Consequently aliquots of the distillate con-

TABLE I  
*Formaldehyde Concentration and Color Intensity*

Reading of unknown	Formaldehyde present	Formaldehyde found	Deviation from known values
72 $\gamma$ standard, set at 20 mm.			
mm.	$\gamma$	$\gamma$	per cent
39.1	36	36.7	+2.0
26.4	54	54.5	+0.9
16.1	90	89.5	-0.6
13.6	108	106.0	-1.5
11.8	126	122.0	-3.0
36 $\gamma$ standard			
38.5	18	18.7	+3.8
13.9	54	51.5	-4.5

taining 50 to 100  $\gamma$  are compared with a standard containing 70 to 75  $\gamma$  at a volume of 50 cc.

*Estimation of Serine*—Table II reveals the per cent recovery of serine alone and in the presence of other amino acids. In most cases the recovery of 1 to 5 mg. of serine was 99 per cent of the theoretical value. Threonine, which forms acetaldehyde with periodate, appears to have no effect upon the yield of serine. Furthermore, when serine is added to collagen hydrolysate, it is completely recovered.

There are indications that on standing cysteine is spontaneously converted in part to serine (Table II). Analysis of old samples of c.p. cysteine hydrochloride available in the laboratory were shown to contain 0.6 to 1.0 per cent serine. Freshly prepared samples made from cystine, on the other hand, yielded only 0.2 per cent serine on analysis.

*Analysis of Protein Hydrolysates*—To test the applicability of the procedure to the determination of serine in proteins, determinations were carried out on the hydrolysates of crystalline hemoglobin and egg albumin, and purified casein, collagen, and salmine. Recent determinations of the serine content of hemoglobin, egg albumin, and salmine were not found in the literature. To test the effect of carbohydrates, mannose, lactose, and glucosamine were added to collagen before hydrolysis. The rate of

TABLE II  
*Estimation of Serine*

Substances other than serine added		Serine added	Serine found	Per cent recovery*
	mg.	mg.	mg.	
	0	1.00	0.98- 0.99	98.5 - 99.2
	0	5.00	4.92- 5.05	98.5 -101.0
	0	50.0	48.80-48.5	97.0 - 97.5
Threonine.....	50.0	5.0	4.95- 4.97	99.0 - 99.3
".....	100.0	5.0	4.91- 4.97	98.2 - 99.3
Glutamic acid.....	50.0	5.00	4.95- 4.97	99.0 - 99.3
Cysteine hydrochloride (old).....	1000	0	10.0	
" " (new).....	1000	0	2.0	
Collagen† 50 mg. ....		0	1.77- 1.79‡	
" 50 " .....		1.0	2.77- 2.83‡	100 -102
" 50 " .....		2.0	3.84- 3.87‡	102 -103
" 50 " + mannose.....	10	0	1.71- 1.72‡	97.0 -97.5
" 50 " + glucosamine....	2.5	0	1.88‡	105
" 50 " + ".....	10	0	2.71- 2.88‡	152 -161
" 50 " .....	0	0	1.88§	
" 50 " + lactose.....	2.5	0	1.90- 1.92§	101 -102

\* Maximum deviation from the theoretical.

† The substances indicated in the table were added to collagen. The mixture was hydrolyzed at 110° with 6.8 N HCl. Aliquots of the hydrolysate which contained before hydrolysis 50 mg. of collagen and the indicated amounts of other substances were taken for analysis.

‡ Hydrolysis for 24 hours.

§ Hydrolysis for 6 hours.

liberation and subsequent destruction of serine by acid hydrolysis was tested on casein and collagen.

The proteins were purified as follows: Twice crystallized oxyhemoglobin was prepared according to the method of Taylor and Hastings (9). Egg albumin was recrystallized three times to eliminate ovomucoid which contains considerable polysaccharide. According to Levene and Mori (10) thrice crystallized ovalbumin contains only 0.26 per cent polysaccharide. Collagen prepared from steer hide by a mild treatment with

alkali was kindly supplied by Dr. J. H. Highberger (11). It may contain approximately 0.5 per cent carbohydrate (glucose and galactose) (12). Casein was prepared from fresh milk by the method of Van Slyke and Baker (13). The filtrate from 0.5 gm. gave a negative test for free reducing sugar. Salmine was a commercial product.

The method for preparing acid hydrolysates was as follows: 1 gm. of protein was placed in a 250 cc. Erlenmeyer flask, followed by the addition of 40 cc. of water and 50 cc. of concentrated HCl. The final concentration of the acid was about 6.8 N. The flask was connected to a reflux condenser

TABLE III  
*Serine Content of Proteins*

Protein	Serine in sample	Total N of sample	Serine N of hydrolysate
	mg.	mg.	per cent total N
Horse Hb*	2.17	6.60	4.38
	2.24	6.60	4.46
Dog Hb*	2.14	6.57	4.30
	3.53	11.30	4.15
Collagen (6 hrs. hydrolysis)	1.88	7.73	3.24
	1.86	7.73	3.20
Egg albumin	4.43	9.45	6.25
	4.46	9.45	6.30
Salmine	2.28	9.33	3.26
	2.26	9.33	3.21
Casein†	2.57	7.14	4.78
	2.47	6.99	4.72

\* We are indebted to Mr. J. R. Braunstein for the preparation of the crystalline hemoglobin.

† Two samples of this casein contained 0.21 mm of organic P and 0.53 mm of serine per gm. After being dephosphorylated by treatment with 0.25 N NaOH at 34° for 48 hours before hydrolysis, it contained 0.33 and 0.34 mm of serine respectively. Nearly 40 per cent of the serine was destroyed. Nicolet, Shinn, and Saidel (15) obtained similar results with silk proteins.

and the contents boiled for 24 hours (unless indicated otherwise). The acid hydrolysate was brought to a volume of 100 cc. and 5 to 10 cc. aliquots analyzed for serine. All hydrolysates were first adjusted to the alkaline side of methyl red before the addition of periodic acid. If the hydrolysate contained humin, the sample was centrifuged before analysis. Nitrogen was determined by the Kjeldahl procedure on all protein samples before hydrolysis. Organic phosphorus in casein was determined by the method of Fiske and Subbarow (14).

Table III gives the serine content of proteins, expressed as per cent nitro-

gen of the total nitrogen. The serine and phosphate content of casein is also given in mm. The result shows that casein contains more than 2 moles of serine per mole of organic phosphorus.

*Liberation and Destruction of Serine by Acid Hydrolysis*—Fig. 1 shows that the serine of casein or collagen after its liberation is slowly destroyed by acid hydrolysis. The highest serine value for casein is obtained at 18 hours hydrolysis. At 24 hours the value is only 1 per cent lower. The highest value for collagen is obtained in 3 hours. At 6 hours the result is 1 per cent lower.

*Effect of Carbohydrates*—Periodic acid reacts with carbohydrates to give formaldehyde. Carbohydrates contained in proteins may affect the serine

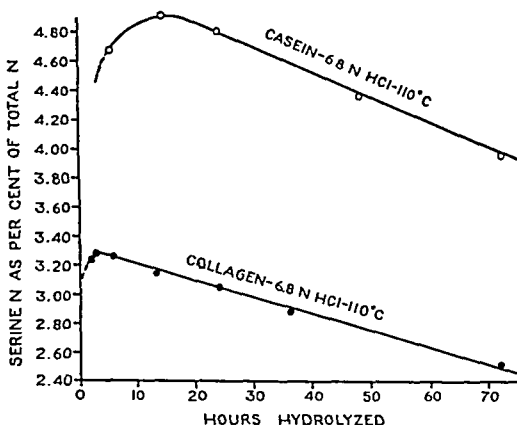


FIG. 1. Liberation and destruction of serine during the hydrolysis of casein and collagen.

determination in the amino acid mixture prepared from them unless the sugar is completely converted to other compounds (presumably furfural derivatives) during the hydrolysis.

Table II shows that mannose added to the extent of 20 per cent of the weight of collagen did not influence the serine estimation of the latter if the protein was hydrolyzed for 24 hours with 6.8 N HCl. Lactose added to the extent of 5 per cent of the weight of the collagen did not influence the results after 6 hours hydrolysis. Nicolet and Shinn (16) have shown that lactose did not increase the yield of formaldehyde if added to casein and subsequently hydrolyzed for 24 hours with 20 per cent HCl. It is therefore reasonable to infer that the determined value of serine in casein (24 hours hydrolysis) or collagen (6 hours hydrolysis) is not appreciably



influenced by the carbohydrate of the proteins. On the other hand (Table II), 1 mole of glucosamine (which is more stable to acid hydrolysis) yields approximately 0.1 mole of formaldehyde when added to collagen and hydrolyzed for 24 hours with 6.8 N HCl. The interference of glucosamine is of such magnitude that the determination of serine in a mucoid might be seriously in error. The carbohydrate content of certain proteins such as thrice crystallized egg albumin (10) is so small, however, that it might be expected to have no influence on the serine determination even though the carbohydrate consisted essentially of glucosamine.

### Comment

The serine values for the protein hydrolysates analyzed range between 3.07 and 6.3 per cent (calculated as per cent N of the total nitrogen). These values include formaldehyde which may be derived from hydroxylysine. However, Van Slyke, Hiller, and MacFadyen (5) have shown that most of the proteins analyzed for hydroxylysine do not contain over 0.1 per cent of hydroxylysine expressed as per cent N of the total nitrogen. Only gelatin or collagen contains as much as 1.0 per cent of hydroxylysine.

### SUMMARY

A colorimetric method for the measurement of serine in small amounts (1 to 5 mg.) has been developed. The formaldehyde which is formed by the action of periodate on serine is quantitatively distilled from the reaction mixture, condensed with 1,8-dihydroxynaphthalene-3,6-disulfonic acid, and measured colorimetrically with an error of not more than 1 or 2 per cent.

The procedure has been applied to known amino acid mixtures, acid hydrolysates of purified casein, collagen, salmine, crystalline egg albumin, and hemoglobin. Liberation and destruction of serine during hydrolysis of proteins have been studied.

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# EFFECT OF METAL IONS ON THE REACTIONS OF PHOSPHO-PYRUVATE BY *ESCHERICHIA COLI*

By M. F. UTTER AND C. H. WERKMAN

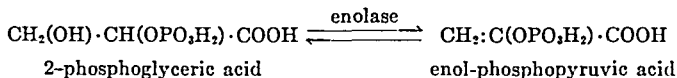
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$Mg^{++}$  and  $Mn^{++}$  were recently observed (1) to facilitate the rapid establishment of the phosphoglyceromutase-enolase equilibrium in a cell-free preparation of *Escherichia coli*. The study of the effect of the metal ions is extended in this communication.

Metal ions, especially  $Mg^{++}$  and  $Mn^{++}$ , are required in many different enzyme systems, particularly in those processes involving phosphorylation. Phosphatases from various sources have been reported to require metal ions for activation. Pett and Wynne (2) noted the effect of  $Mg^{++}$  on the phosphatase action of bacteria.  $Mg^{++}$ , in most cases replaceable by  $Mn^{++}$ , is also known to play an important part in the transfer of phosphate from phosphopyruvate to creatine via adenylic acid (3), and from phosphopyruvate to glucose (4). At least one case of intramolecular transfer, the conversion of glucose-1-phosphate to glucose-6-phosphate, is known to require  $Mg^{++}$ ,  $Mn^{++}$ , or  $Co^{++}$  (5). Metal ions have been reported as integral parts of the following enzymatic reactions: the decarboxylation of pyruvate by yeast (6), oxidation of pyruvate (7), the formation of acetylmethylcarbinol (8), decarboxylation of oxalacetate (9), and the oxidation of glucose (10). This is by no means a complete list of reactions requiring metal ions.

The metal stimulation in the present case has been found to occur in the enolase reaction.



It is interesting to note that this reaction involves a dehydration and does not constitute an intramolecular phosphate transfer. The effect of metal ions upon the transfer of phosphate to adenylic acid and the dephosphorylation of adenosine triphosphate is also considered in this report.

## Methods

The enzyme system used in most of these experiments was prepared from *Escherichia coli* by grinding the cell paste with powdered glass as described in previous reports (1, 11). After clarification on the Beams ultracentrifuge (12), the extract was rapidly dried *in vacuo*. The resulting powder could

be stored in the ice box for a period of at least 4 weeks without appreciable loss of activity. The experimental preparations were secured by dissolving 30 mg. of the dried powder per ml. of distilled water. This solution was then dialyzed in a collodion tube at 4° for 3 to 4 hours against circulating distilled water.

In one experiment Lebedev extract was substituted for the bacterial preparation. 25 gm. of yeast were incubated for 3 hours at 37° in 75 ml. of water; the solution was then centrifuged on an angle centrifuge until the main portion of the solids had been removed. The juice was then further clarified on the Beams ultracentrifuge, dialyzed for 5 hours against distilled water, and again centrifuged briefly on the ultracentrifuge.

Inorganic phosphate was determined with a Klett-Summerson photometer by the method of Fiske and Subbarow (13). Phosphopyruvate was determined as the fraction hydrolyzable in alkaline iodine solution at room temperature (14). Adenosine triphosphate was determined as the phosphate fraction hydrolyzed by *N* HCl in 7 minutes at 100°, or in the presence of phosphopyruvate as the difference between the 60 minute hydrolyzable fraction (adenosine triphosphate + phosphopyruvate) minus the alkaline iodine fraction (phosphopyruvate). Phosphoglycerate was determined as the difference between the total phosphate (wet ash) and the 180 minute hydrolyzable fraction. Triose phosphate (glyceraldehyde phosphate and dihydroxyacetone phosphate) was determined as the phosphate fraction hydrolyzed at room temperature in 20 minutes by *N* NaOH (15). Hexose diphosphate was determined by ashing (in the absence of other organic phosphates).

The experiments were performed in the presence of air, since the bacterial preparation has negligible aerobic activity. The reactions do not proceed beyond phosphopyruvate to an appreciable extent unless a phosphate acceptor is added. The experiments were carried out in calibrated test-tubes and the reaction was stopped at the end of the incubation period by adding 5 ml. of 4 per cent  $\text{CCl}_3\text{COOH}$ . The solution was diluted to 10 ml., filtered, and the various determinations were run on aliquots of the filtrate. Duplicate tubes in which the deproteinizing agent was added at the start of the experiment served as controls.

Na hexose diphosphate, Na phosphoglycerate, and Na adenosine triphosphate were prepared from the Ba salts of the compounds. The sodium salt of adenylic acid was prepared by dissolving and neutralizing the free acid. Dr. Gerhard Schmidt kindly supplied synthetic Ag-Ba phosphopyruvate from which the Na salt was prepared.

#### EXPERIMENTAL

Inasmuch as it has been found that the percentage stimulation decreases as equilibrium is approached, it was necessary to stop the action before

equilibrium is reached in order to make apparent the metal ion stimulation of the phosphoglyceromutase-enolase reaction. Two factors control the rapidity with which the equilibrium is reached; *i.e.*, enzyme concentration and substrate concentration. The enzyme concentration was kept relatively low and the substrate concentration relatively high for the purpose of these experiments. The rate of stimulation by the metal ions is shown to decrease (Table I) as the reaction approaches equilibrium. The figures in the last column represent the ratio of the rates of the stimulated reaction to the unstimulated. This ratio decreases as the reaction progresses, and would be 1.0 if both reactions reached equilibrium, where the theoretical value of 30 per cent phosphoglycerate is converted into phosphopyruvate.

TABLE I

*Effect of Time on Stimulation of Phosphoglyceromutase-Enolase Equilibrium by  $Mg^{++}$*

The enzyme preparation was dialyzed 3 hours. For each experiment the following were used: 0.1 ml. of enzyme (3 mg. dry weight), 0.06 M  $NaHCO_3$  freshly saturated with  $CO_2$ ,  $41.2 \times 10^{-2}$  mg. of phosphoglycerate P, a total volume of 1.0 ml., temperature  $25^\circ$ .

Experiment No.	Time	Final concentration of $MgCl_2$	Phosphopyruvate P, $1 \times 10^{-2}$	Phosphoglycerate converted	Ratio of stimulated to unstimulated
	<i>min.</i>	<i>M</i>	<i>mg.</i>	<i>per cent</i>	
1	5		0.733	1.78	
2	5	0.005	2.467	5.99	3.365
3	10		1.533	3.72	
4	10	0.005	5.200	12.62	3.392
5	20		3.333	8.09	
6	20	0.005	6.933	16.83	2.080
7	40		4.800	11.65	
8	40	0.005	8.533	20.71	1.778
9	60		6.200	15.05	
10	60	0.005	9.800	23.79	1.581

Owing to deterioration of the catalyst, equilibrium in a weak enzyme solution, such as that employed, is reached with difficulty. The concentration of  $MgCl_2$  (0.005 M) used in the experiments in Table I is approximately optimum, as shown in Fig. 1 in which various concentrations of  $MgCl_2$  and  $MnSO_4$  have been used. The optimum concentration of the two ions is similar and the stimulations are comparable. Although  $Mg^{++}$  appears to be slightly more efficient than  $Mn^{++}$  in this particular experiment, the converse also has been observed. It will be noted that both ions are inhibitory at the higher concentrations.

$Mn^{++}$  and  $Mg^{++}$  are the only effective ions in the group under observation. In the experiments in Table II, several other ions have been found to be without effect or to be inhibitory. The stimulatory effect of  $Mg^{++}$

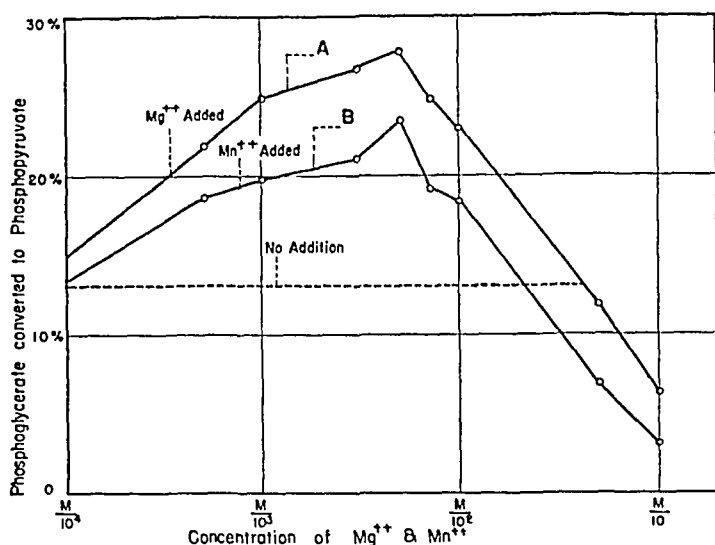


FIG. 1. Effect of the concentration of  $\text{MgCl}_2$  and  $\text{MnSO}_4$  on the phosphoglyceromutase-enolase equilibrium. For each experiment the following were used: 0.2 ml. of extract dialyzed 3 hours, 0.06 M  $\text{NaHCO}_3$ ,  $41.2 \times 10^{-2}$  mg. of phosphoglycerate P, a total volume of 1.0 ml. Incubation was for 15 minutes at  $25^\circ$ .

TABLE II

*Effect of Various Metals on Phosphoglyceromutase-Enolase Equilibrium*

The enzyme extract was dialyzed 3 hours. For each experiment the following were used: 0.1 ml. of enzyme extract, 0.06 M  $\text{NaHCO}_3$ ,  $41.2 \times 10^{-2}$  mg. of phosphoglycerate P, a total volume of 1.0 ml. Incubation was for 20 minutes at  $25^\circ$ .

Experiment No.	Additions in final concentration	Phosphopyruvate P formed, $1 \times 10^{-2}$	Phosphoglycerate converted	Experiment No.	Additions in final concentration	Phosphopyruvate P formed, $1 \times 10^{-2}$	Phosphoglycerate converted
	M	mg.	per cent		M	mg.	per cent
1	None	2.867	6.96	9	0.0001 $\text{NiCl}_2$	2.267	5.50
2	0.005 $\text{MgCl}_2$	5.867	14.24	10	0.1 $\text{CaCl}_2$	0	0
3	0.005 $\text{MnSO}_4$	6.400	15.53	11	0.01 "	0.200	0.49
4	0.1 $\text{NaCl}$	2.533	6.15	12	0.001 "	1.333	3.24
5	0.01 "	2.733	6.63	13	None	2.733	7.32
6	0.001 "	2.533	6.15	14	0.03 $\text{KCl}$	2.933	7.86
7	0.01 $\text{NiCl}_2$	0.733	1.78	15	0.01 "	2.800	7.50
8	0.001 "	1.867	4.53	16	0.005 "	2.600	6.96

and  $\text{Mn}^{++}$  on the transfer of phosphate from phosphopyruvate to glucose was reported by Ohlmeyer and Ochoa (16) to be in part a reversal of an  $\text{Na}^+$  inhibition.  $\text{K}^+$  and  $\text{NH}_4^+$  also had the power of reversing the inhibition in

the experiments of Ohlmeyer and Ochoa, although higher concentrations of the latter ions were required. In the present experiments,  $K^+$  (Experiments 14 to 16) is seen to be without appreciable influence; so that the effect is not analogous to that reported by Ohlmeyer and Ochoa.  $Na^+$  is also without effect but  $Ca^{++}$  and  $Ni^{++}$  are inhibitory.

It is reported that generally  $Mg^{++}$  and  $Mn^{++}$  are interchangeable in their effect on phosphate transfer and other reactions although either ion may be more efficient than the other. In a few cases other ions have been found to replace  $Mg^{++}$  and  $Mn^{++}$ ; in carboxylase  $Cd^{++}$ ,  $Ni^{++}$ , and  $Co^{++}$  are also active (17) and in some of the phosphatases  $Fe^{++}$ ,  $Co^{++}$ ,  $Ni^{++}$ , and  $Cd^{++}$  have been found to be effective (18).

TABLE III

*Effect of  $MgCl_2$  and  $MnSO_4$  on Phosphoglyceromutase-Enolase Equilibrium in Lebedev Extract*

The Lebedev extract was dialyzed 5 hours, then recentrifuged. For each experiment the following were used: 0.1 ml. of enzyme, 0.06 M  $NaHCO_3$ ,  $41.2 \times 10^{-2}$  mg. of phosphoglycerate P, a total volume of 1.0 ml. Incubation was for 7 minutes at 23°.

Experiment No.	Final concentration of $MgCl_2$	Phospho-pyruvate P, $1 \times 10^{-2}$	Phospho-glycerate converted	Experiment No.	Final concentration of $MnSO_4$	Phospho-pyruvate P, $1 \times 10^{-2}$	Phospho-glycerate converted
	M	mg.	per cent		M	mg.	per cent
1	None	4.667	11.33	9	None	4.667	11.33
2	0.1	2.067	5.02	10	0.1	1.600	3.88
3	0.05	3.600	8.74	11	0.05	1.533	3.72
4	0.01	7.533	18.28	12	0.01	9.200	22.33
5	0.007	8.067	19.58	13	0.007	9.667	23.46
6	0.005	8.866	21.52	14	0.005	9.133	22.17
7	0.001	7.333	17.80	15	0.001	7.333	17.80
8	0.0005	5.733	13.92	16	0.0005	6.200	15.05

The action of  $Mg^{++}$  and  $Mn^{++}$  on the phosphoglyceromutase-enolase equilibrium in Lebedev extract is demonstrated (Table III) in order to show that the stimulation is not limited to bacterial preparations. Because of the higher enzyme concentration of the Lebedev extract, and consequently the rapidity with which the equilibrium is reached, the demonstration of the stimulation is more difficult. The Lebedev extract was treated as described under "Methods" and a small quantity was employed for a short period of time. Under these conditions, the stimulation can be readily observed, and is similar to that in the bacterial extract. The optimum concentration of the ions appears to be near 0.005 M, and the higher concentrations of the ions used were inhibitory.  $Mn^{++}$  seems to be slightly more efficient in this case. The effect of the metals on Lebedev extract is analogous to that on the bacterial preparation.



In regard to the inhibition by higher concentrations of  $Mg^{++}$ , it has been reported previously that the equilibrium between hexose diphosphate and triose phosphate is displaced toward hexose diphosphate by high concentrations of  $Mg^{++}$  (19). In view of this fact, the reaction was reinvestigated in the lower concentration ranges of the ions in order to determine whether they have a stimulatory action. Results indicate that the effect of the ions is limited to an inhibitory action at high concentrations. Iodoacetate was added to prevent the reaction from proceeding beyond the triose phosphate stage, since dialysis for 3 to 4 hours is probably not sufficient to remove all coenzyme I from the preparation. In the presence of the coenzyme the triose phosphate products of the equilibria are removed by the oxidation-reduction reaction.

Although  $Mg^{++}$  accelerates attainment of the phosphoglyceromutase-enolase equilibrium, it does not change the final point of equilibrium. Re-

TABLE IV

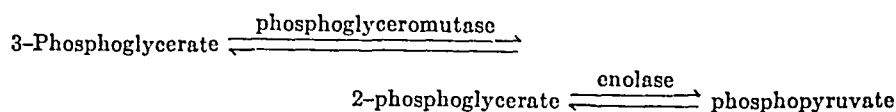
*Effect of  $MgCl_2$  on Final Point of Equilibrium of Phosphoglyceromutase-Enolase System*

The enzyme extract was dialyzed 3 hours. For each experiment the following were used: 0.2 ml. of extract, 0.06 M  $NaHCO_3$ ,  $18.67 \times 10^{-2}$  mg. of phosphoglycerate P, a total volume of 1.0 ml., temperature  $24^\circ$ .

Experiment No.	Time	Final concentration of $MgCl_2$	Phosphopyruvate P, $1 \times 10^{-2}$	Phosphoglycerate converted	Ratio of stimulated to unstimulated
	min.	M	mg.	per cent	
1	5		0.667	3.57	2.80
2	5	0.005	1.867	10.00	
3	10		2.000	10.71	
4	10	0.005	2.933	15.71	1.47
5	65		5.667	30.35	
6	65	0.005	5.533	29.64	0.98

sults in Table IV show that when sufficient time has been allowed for the establishment of the equilibrium in the presence and in the absence of  $Mg^{++}$  the theoretical value of 30 per cent phosphopyruvate is obtained in both cases.

Previous experiments have not indicated which of the two enzymes involved in the reaction is stimulated by the metal ions. The entire equilibrium system is as follows:



In the experiments reported in Fig. 2, phosphopyruvate, rather than phosphoglycerate, was used as the substrate. An optimum concentration

of  $Mg^{++}$  was present in the experiments denoted by Curve A, and with no  $Mg^{++}$  added in the experiments represented by Curve B.

As shown in a previous report (1) and as determined by Meyerhof and Schulz (20), the entire equilibrium reveals the following approximate distribution among the three compounds: 3-phosphoglycerate 63.5 per cent, 2-phosphoglycerate 6.5 per cent, phosphopyruvate 30 per cent. The ratios between 3- and 2-phosphoglycerate and between 2-phosphoglycerate and phosphopyruvate are not affected by the absence of the third component; i.e., the equilibrium between 2-phosphoglycerate and phosphopyruvate (without 3-phosphoglycerate) is established when about 17 per cent of the

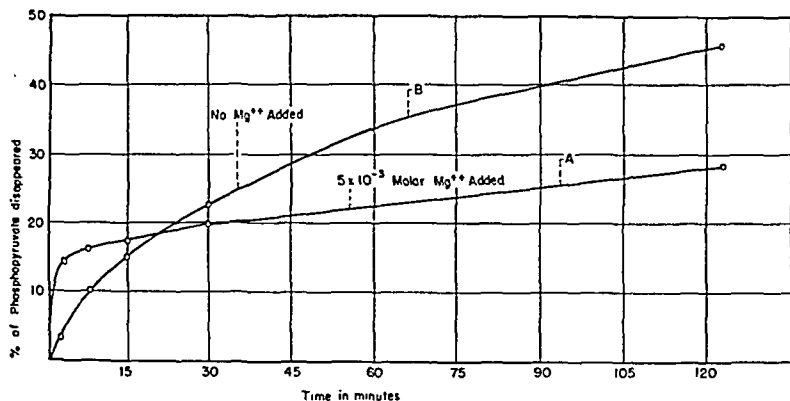


FIG. 2. Effect of  $MgCl_2$  on the conversion of phosphopyruvate to phosphoglycerate. For each experiment the following were used: 0.2 ml. of extract dialyzed 3 hours, 0.06 M  $NaHCO_3$ ,  $9.866 \times 10^{-2}$  mg. of phosphopyruvate P, a total volume of 1.0 ml., temperature  $25^\circ$ . In the experiments denoted by Curve A an optimum concentration of  $Mg^{++}$  was present; in those of Curve B no  $Mg^{++}$  was added.

phosphopyruvate has disappeared. If an equilibrium involving all three components were established, 70 per cent of the phosphopyruvate would disappear. A sharp stimulation by the  $Mg^{++}$ -enzyme preparation occurs in comparison to the effect of the enzyme alone (Fig. 2) until the theoretical value of 17 per cent of phosphopyruvate converted into phosphoglycerate is approached. After this point is reached, the  $Mg^{++}$ -enzyme curve breaks sharply, and is actually surpassed by the unstimulated enzyme after 30 minutes, which leads one to believe that the  $Mg^{++}$  facilitates a rapid establishment of the enolase equilibrium with negligible formation of 3-phosphoglycerate but that thereafter the  $Mg^{++}$  actually inhibits the subsequent conversion of the 2-phosphoglycerate into the 3 form. This interpretation

is borne out by the sharp break in the  $Mg^{++}$ -enzyme curve just below the value of 17 per cent phosphopyruvate "disappeared." On the other hand, the unstimulated enzyme shows neither the stimulation of the enolase reaction nor the inhibition of the phosphoglyceromutase enzyme, and the resulting curve is relatively smooth.

As further evidence of the stimulation of the enolase reaction by the metals, it was noted that in the experiments of Table V, in which pure 3-phosphoglycerate<sup>1</sup> was used instead of the usual mixture of 3 and 2 isomers, the stimulatory effect of the metals was much reduced. Akano (21) showed that phosphoglyceromutase was much more sensitive to treatment such as dialysis or aging than enolase. Treatment of the bacterial preparations in these experiments undoubtedly had a similar effect, since the extract used underwent a somewhat more rigorous treatment than in the majority of the

TABLE V

*Effect of  $MgCl_2$  on Conversion of Recrystallized 3-Phosphoglycerate to Phosphopyruvate*

The extract was dialyzed 4 hours, then frozen in an ice box overnight. For each experiment the following were used: 0.1 ml. of extract, 0.06 M  $NaHCO_3$ , a total volume of 1.0 ml. Incubation was for 20 minutes at 26°.

Experiment No.	Type of phosphoglycerate	Phospho-glycerate P, $1 \times 10^{-2}$	Final concentration of $MgCl_2$	Phospho-pyruvate P formed, $1 \times 10^{-2}$	Phospho-glycerate converted
		mg.	M	mg.	per cent
1	Normal mixture	37.33		0.733	1.96
2	" "	37.33	0.005	2.000	5.36
3	3-Phosphoglycerate	48.10		0.933	1.94
4	"	48.10	0.005	1.133	2.36

other experiments. The time of dialysis was greater and the extract was not used until the day following dialysis although it was frozen during the intervening period. If the phosphoglyceromutase is more severely affected than the enolase, the activity of the phosphoglyceromutase rather than the metal content of the enolase becomes the limiting factor of the reaction and the metal stimulation is lowered. With the normal mixture of 3- and 2-phosphoglycerates containing about 10 per cent of the 2 isomer, the  $Mg^{++}$  stimulation is much more noticeable because of the immediate conversion of 50 per cent or more of the original 2-phosphoglycerate into phosphopyruvate. In connection with these experiments, it should be mentioned that undialyzed extracts readily attacked pure 3-phosphoglycerate.

The enolase reaction has long been known to be inhibited by NaF. Mas-

<sup>1</sup> The pure 3-phosphoglycerate was obtained by recrystallization from the mixture of 3 and 2 isomers as described in a previous paper (1).

sart (22) has suggested that the inhibition of succinodehydrogenase by NaF and Na pyrophosphate is due to the removal of metal ions necessary for the reaction. Massart and Dufait (23) have further suggested that the inhibition of certain phosphatases by NaF is due to a similar formation of a metal complex. Experiments concerning the effect of NaF upon the enolase reactions were undertaken to determine whether the inhibitions are analogous.<sup>2</sup>

The effect of NaF upon the conversion of phosphopyruvate to phosphoglycerate is shown in Table VI. In the case of metal addition the enzyme preparations were incubated for a short time with the metals before the addition of the substrate and inhibitor. NaF is seen to inhibit the unstimu-

TABLE VI

*Effect of NaF, MgCl<sub>2</sub>, and MnSO<sub>4</sub> on Transformation of Phosphopyruvate to Phosphoglycerate*

The extract was dialyzed 3 hours. For each experiment the following were used: 0.2 ml. of extract, 0.06 M NaHCO<sub>3</sub>,  $11.6 \times 10^{-2}$  mg. of phosphopyruvate P, a total volume of 1.0 ml. Incubation was for 5 minutes at 25°. In experiments in which other additions were made MgCl<sub>2</sub> = 0.015 M, MnSO<sub>4</sub> = 0.015 M, NaF = 0.04 M.

Experiment No.	Additions	Phosphopyruvate P, $1 \times 10^{-2}$	Inorganic P, $1 \times 10^{-2}$	Total organic P, $1 \times 10^{-2}$	Phosphopyruvate P converted to phosphoglycerate, $1 \times 10^{-2}$	Phosphopyruvate converted
		mg.	mg.	mg.	mg.	per cent
1	None	10.733	0.266	11.334	0.601	5.30
2	NaF	11.600	0	11.600	0	0
3	MgCl <sub>2</sub>	9.400	0.400	11.200	1.800	16.07
4	" + NaF	11.600	0	11.600	0	0
5	MnSO <sub>4</sub>	9.933	0.333	11.267	1.334	11.84
6	" + NaF	10.867	0	11.600	0.733	6.32

lated enzyme and the Mg<sup>++</sup>-enzyme completely but merely to decrease the activity of the Mn<sup>++</sup>-enzyme preparation. Massart and Dufait (23) found a somewhat similar situation with yeast phosphatase and gave the explanation that the Mn<sup>++</sup>-NaF complex is much more unstable than the Mg<sup>++</sup>-NaF complex.

In a previous paper (1) the transfer of PO<sub>4</sub> from phosphopyruvate to adenylic acid was demonstrated by means of an extract of *Escherichia coli* but the effect of metal ions upon the reaction was not observed. In Table VII the effect of Mg<sup>++</sup>, Mn<sup>++</sup>, and NaF upon the PO<sub>4</sub> transfer is tabulated. In Experiment 1, in which there is no NaF addition, the phosphopyruvate

<sup>2</sup> We wish to express our appreciation to Mr. Krampitz for suggestions in regard to this phase of the work.

which cannot be accounted for as residual phosphopyruvate (12.0 - 9.967), adenosine triphosphate, or inorganic phosphate is in the form of phosphoglyceric acid, since the enolase reaction can take place in the absence of NaF. When the inhibitor is added (Experiment 3), most of the phosphopyruvate can be accounted for. Adenylic acid without metal ions can accept  $\text{PO}_4$  to a limited extent (Experiment 4) but NaF stops the formation of adenosine triphosphate.  $\text{Mg}^{++}$  greatly stimulates the formation of adenosine triphosphate but again NaF stops the reaction (Experiment 7).  $\text{Mn}^{++}$  also effectively stimulates the formation of adenosine triphosphate but NaF causes only a slight decrease in the  $\text{PO}_4$  transfer. This case, then,

TABLE VII

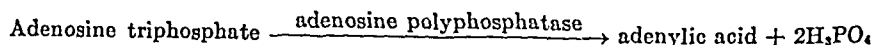
*Effect of  $\text{MgCl}_2$ ,  $\text{MnSO}_4$ , and NaF on Transfer of Phosphate from Phosphopyruvate to Adenylic Acid*

The extract was dialyzed 3 hours. For each experiment the following were used: 0.2 ml. of extract, 0.06 M  $\text{NaHCO}_3$ ,  $12.00 \times 10^{-2}$  mg. of phosphopyruvate P, a total volume of 1.1 ml. Incubation was for 30 minutes at  $26^\circ$ . Molar values for experiments in which additions were made were NaF 0.08 M, adenylic acid (AA) 0.005 M,  $\text{MnSO}_4$  and  $\text{MgCl}_2$  0.005 M. Values are given in  $\text{P} \times 10^{-2}$  mg. per 1.1 ml.

Experiment No.	Additions	Residual phosphopyruvate	Phosphopyruvate disappeared	60 min. hydrolyzable phosphorus	Adenosine triphosphate	Change in inorganic P	Phosphopyruvate accounted for
1	Control, AA added, not incubated	12.000	0	12.133	0.133	0	12.133
2	Control, AA not added, incubated	9.267	2.733	9.733	0.567	0.133	9.967
3	NaF	11.667	0.333	11.867	0.200	0	11.867
4	AA	6.800	5.200	10.533	3.733	0.733	11.266
5	" NaF	11.600	0.400	11.533	-0.067	0	11.533
6	$\text{MgCl}_2$ , AA	0.467	11.533	10.133	9.663	2.067	12.197
7	" " NaF	11.133	0.867	12.000	0.867	0	12.000
8	$\text{MnSO}_4$ , "	1.333	10.667	11.133	9.800	0.933	12.066
9	" " NaF	3.067	8.933	10.400	7.333	0.800	11.200

is analogous to that of enolase in which  $\text{Mn}^{++}$  stimulation is not removed by NaF, while  $\text{Mg}^{++}$  stimulation disappears when the inhibitor is added. In another experiment  $\text{K}^+$  was observed to have no effect; thus the stimulation is not a reversal of an  $\text{Na}^+$  inhibition.

To study the effect of  $\text{Mg}^{++}$  and  $\text{Mn}^{++}$  upon adenosine polyphosphatase, less centrifugation was employed in the preparation of the extract, since this enzyme is almost entirely removed by prolonged centrifugation. This enzyme catalyzes the following reaction.



The extract used in this experiment has a much higher enzyme content than the preparations used in the previous experiments and was used without drying. The stimulatory effects of  $Mg^{++}$  and  $Mn^{++}$  upon the phosphatase action are recorded in Table VIII. Both ions have distinct effects but NaF removes most of the effects of  $Mg^{++}$  (Experiment 14), and also that of  $Mn^{++}$  (Experiment 15), contrary to the experience with  $Mn^{++}$  and NaF with enolase and the  $PO_4$ -transferring enzyme. It should be mentioned again that the enzyme preparation used in this experiment is not identical to those used in the enolase and  $PO_4$ -transferring experiments and that this fact may be responsible for the difference in results. A longer dialysis period (5.5 hours) failed to change the essential effect of the  $Mg^{++}$ ,  $Mn^{++}$ ,

TABLE VIII

*Effect of Metals and NaF on Dephosphorylation of Adenosine Triphosphate*

The extract was dialyzed 3 hours. For each experiment the following were used: 0.2 ml. of extract, 0.06 M  $NaHCO_3$ ,  $9.68 \times 10^{-2}$  mg. of adenosine triphosphate P, a total volume of 1.0 ml. Incubation was for 60 minutes at  $24^\circ$ .

Experiment No.	Additions, final concentration	Inorganic P liberated, $1 \times 10^{-3}$	Ratio of stimulated to unstimulated	Experiment No.	Additions, final concentration	Inorganic P liberated, $1 \times 10^{-3}$	Ratio of stimulated to unstimulated
	<i>M</i>	mg.			<i>M</i>	mg.	
1	None	1.667		9	None	1.667	
2	0.01 $MgCl_2$	6.133	3.68	10	0.0005 $MnSO_4$	8.200	4.92
3	0.005 "	5.933	3.56	11	0.075 KCl	1.333	0.80
4	0.001 "	7.733	4.64	12	0.015 "	1.800	1.08
5	0.0005 "	6.400	3.84	13	0.0075 "	1.733	1.04
6	0.01 $MnSO_4$	2.000	1.20	14	0.005 $MgCl_2$ , 0.04 NaF	2.200	1.32
7	0.005 "	3.267	1.96	15	0.005 $MnSO_4$ , 0.04 NaF	1.333	0.80
8	0.001 "	6.067	3.64	16	0.04 NaF	1.333	0.80

and NaF, but the activity in the absence of the metals was very low; therefore the percentage of stimulation was much larger.

SUMMARY<sup>3</sup>

1. In an extract prepared by grinding *Escherichia coli* with powdered glass,  $Mg^{++}$  and  $Mn^{++}$  are shown to accelerate attainment of the phosphoglyceromutase-enolase equilibrium without changing the point of final equilibrium.  $K^+$  and  $Na^+$  have no effect and  $Ca^{++}$  and  $Ni^{++}$  are inhibitory. The effect on Lebedev extract is similar.

<sup>3</sup> Since submission of this manuscript, an abstract (24) of the papers by Warburg and Christian (25) has come to our attention. They succeeded in crystallizing enolase and report it has a metallic component, probably  $Mg^{++}$ .

2. The effect of  $Mg^{++}$  and  $Mn^{++}$  on the hexose diphosphate-triose phosphate equilibrium is limited to an inhibitory action in the high concentration range.

3. Enolase is shown to be the enzyme actually stimulated by the metal ions. The inhibition of this enzyme by NaF appears to be linked with the metal component of this system. NaF completely removes  $Mg^{++}$  stimulation but has much less effect on  $Mn^{++}$  stimulation.

4.  $Mg^{++}$  and  $Mn^{++}$  are shown to stimulate the transfer of  $PO_4$  from phosphopyruvate to adenylic acid in the bacterial extract. NaF inhibits the stimulation by  $Mg^{++}$  but not that by  $Mn^{++}$ .

5.  $Mg^{++}$  and  $Mn^{++}$  stimulate the breakdown of adenosine triphosphate in the *Escherichia coli* extract.

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# THE OXYGEN CONSUMPTION, CREATINE, AND CHLORIDE CONTENT OF MUSCLES FROM VITAMIN E-DEFICIENT ANIMALS AS INFLUENCED BY FEEDING $\alpha$ -TOCOPHEROL\*

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(Received for publication, August 22, 1942)

The paralysis in suckling young rats of vitamin E-deficient mothers, first described by Evans and Burr (1) and shown to be preventable by administration of vitamin E, was demonstrated by Olcott (2) to be due to muscle lesions similar to those found in young guinea pigs and rabbits (3) and in ducklings (4) on vitamin E-deficient diets. Hamsters develop the same dystrophic condition on such diets (5) as do older rats (6), and the effectiveness of tocopherol in preventing or curing the disorder (except in old rats) has been demonstrated too often to require reference.

The histological changes seen in such degenerated muscles are accompanied by alterations in chemical composition and in functional behavior. Among the chemical changes perhaps the most striking is the diminished creatine content (7), which is roughly proportional to the degree of degeneration. There is also a decrease in total solids, in total nitrogen, in glycogen, and in total acid-soluble phosphorus and phosphocreatine phosphorus, an increase in cholesterol content (8), a gain in sodium chloride with corresponding loss in potassium and magnesium, and finally an increase in calcium (9, 10). These changes are not necessarily parallel; creatine content may be normal when the chlorides are high (11, 12); creatine may not decrease until dystrophy is extreme and while calcification is still negligible.

The appearance of large quantities of creatine in the urine of rabbits is indicative of the onset of dystrophy (13) but the degree of creatinuria is not proportionate to the severity of dystrophy. The creatine-creatinine ratio in the urine of rabbits has recently been proposed (14) as a simple and reliable method for determining the state of dystrophy. Oral administration of  $\alpha$ -tocopherol or its acetate causes the urinary creatine excretion to drop to a constant level within a few days (13-15).

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† These and the following studies are taken from a dissertation submitted by O. Boyd Houchin to the Faculty of the Graduate College of the State University of Iowa in partial fulfillment of the requirements for the degree of Doctor of Philosophy, July, 1942.



The functional changes in such dystrophic rat muscle include decreased contractile power without marked histological alteration (11); an increased oxygen uptake ( $Q_{O_2}$ ) has been demonstrated in dystrophic muscle from rabbits, guinea pigs, and rats (16-18) and recently from the hamster (5). It is thus obvious that deprivation of vitamin E leads to some alteration in the metabolism of muscle at rest as well as during contraction; although the susceptibility to the lack of vitamin E may appear to differ in these species and with the age of the animals, the uniform physiological alteration suggests that vitamin E may play a hitherto unsuspected rôle in the regulation of oxidative processes in muscle.

The work reported in this and following papers was undertaken to secure further information on the manner in which tocopherol intervenes in the biological processes of oxidation in muscle, on its relation to changes in creatine and chloride in muscle, and on the enzyme systems, if any, in which it is concerned.

#### EXPERIMENTAL

The commonly used dystrophy-producing diet for rabbits, which is low in fat, contained the following ingredients: cellophane 20, casein 15, sucrose 10, starch 36, lard 3, cod liver oil 3, yeast 10, and salt mixture<sup>1</sup> 3 per cent. When the rabbits weighing 500 to 850 gm. were gradually transferred to this diet, signs of muscular dystrophy became apparent in 2½ to 5 weeks. A rabbit was used for study when it was unable to rise after being placed on its side.

The vitamin E-deficient diet given to rats was high in fat and consisted of sucrose 45.5, lard 22, casein 18, yeast 10, salt mixture<sup>1</sup> 2.5, and cod liver oil 2 per cent. The older rats had been placed on this diet at 16 to 20 days of age. The paralyzed suckling rats owed their existence to adequate doses of tocopherol given to their mothers reared on the deficient diet; paralysis developed between the 16th and 25th days after birth.

Control animals were partly from stock diet (Friskies), partly from one of the deficient diets containing 3 to 5 per cent of wheat germ oil in place of lard; some were fed  $\alpha$ -tocopherol acetate or lettuce in addition to the stock diet.

Both of the experimental diets were used with the golden Syrian hamster and this little animal proved to be very susceptible to the lack of vitamin E. Of 51 animals placed on the high fat diet at 21 days of age and 30 to 40 gm. in weight, thirty-eight died within 3 weeks, usually very suddenly, and without appreciable loss of weight. External symptoms were a purulent excretion from the eyes, abnormally pink skin, muscular weakness, and

<sup>1</sup> Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 14, 273 (1937)

sometimes paralysis. Autopsy and microscopic examination<sup>2</sup> revealed respiratory trouble and the typical degeneration of muscle tissue. Of thirty-eight animals on the low fat diet, all died within 2 weeks, with severe dystrophy. On the control diets the animals grew and remained normal.

The oxygen consumption of muscle slices was measured in oxygen at 38° by the usual Warburg procedure in a modified Locke's solution. The semitendinosus muscle and sometimes the gastrocnemius muscle were used. The rats and hamsters were killed by a blow on the head and the rabbits were anesthetized. The muscle was immediately removed and sliced by a special technique: after being cut in lengths and freed from fascia, it was laid on a clean wooden block, held down firmly with a microscope slide, and sliced lengthwise of the structure by a sharp razor blade worked lightly and flat against the under side of the slide. Slices thus obtained were from 0.1 to 0.7 mm. in thickness, sufficiently thin to permit proper diffusion of oxygen, yet without excessive destruction of cells. The results compared favorably with those obtained on muscle strips. The small muscles of the nursing rats were teased out. The oxygen consumption was measured for the 1st hour. The slices were washed with distilled water and dried at 110° to constant weight. Analyses were run in triplicate (or more) when possible. The results are expressed as  $Q_0$ , (c.mm. of oxygen taken up per mg. of dry weight of tissue per hour).

Total creatine was determined by the method of Rose, Helmer, and Chanutin (19), chlorides by the method of Sunderman and Williams (20).

#### DISCUSSION

The data obtained from 59 normal and dystrophic rabbits, rats, and hamsters are summarized in Table I and leave no doubt that dystrophic muscle from vitamin E-deficient animals, as compared with muscle from normal animals, has a higher oxygen consumption, a lower creatine content, and a higher chloride content.

The most striking increases in oxygen consumption are found in the dystrophic hamster on a low fat diet, 240 to 250 per cent of the normal. In rabbits the figure is only slightly less, 220 per cent of the normal, in nursing rats 160 per cent, and in the grown rats 125 per cent. These increases are not related to the intensity of the normal oxygen uptake, which is greatest in the rats and lowest in the rabbits, but rather to the acuteness and severity of the dystrophic condition. When rats from vitamin E-deficient mothers successfully weather the nursing period or spontaneously recover, for reasons as yet unknown, they live for many

<sup>2</sup> The interest and help of Dr. E. D. Warner, of the Department of Pathology, is gratefully acknowledged.

months on the deficient diet. At 6 months they present no external symptoms and only slightly abnormal histology. Even their nursing period is apparently not as critical as the adolescent period of rabbits and hamsters, in which vitamin E is indispensable.

The decreased creatine content of the muscles of vitamin E-deficient rats, only 9 per cent, also reflects the relatively slow alteration produced in this species by a lack of vitamin E. In the rabbit the decrease is some-

TABLE I  
*Average Oxygen Consumption and Creatine and Chloride Content of Skeletal Muscle*

The ranges are given in parentheses.

No. of animals and status	Average weight	$O_{O_2}$	Creatine	Chlorides
	gm.	c.mm. $O_2$	mg. per cent	mg. per cent
5 rabbits, stock diet	1265	1.40 (1.27-1.52)	512 (468-535)	54 (48-64)
12 " vitamin E-deficient	804	3.12 (2.22-3.88)	412 (281-549) (11)*	152 (113-228) (9)*
4 rats, stock diet	297	1.96 (1.88-2.08)	455 (447-469) (3)*	47 (45-49) (3)*
6 " vitamin E-deficient (6 mos. old)	277	2.48 (1.62-3.73)	416 (390-460)	66 (60-77)
2 nursing rats, normal	38	1.88 (1.77-1.98)		
8 " " partly paralyzed	32	3.07 (1.93-4.03)		
8 hamsters, stock diet	95	1.66 (1.05-2.07)		57 (50-61)
2 " deficient diet + vitamin E	93	1.79 (1.78-1.81)		54 (50-58)
6 hamsters, deficient diet high in fat	74	3.02 (2.61-3.66)		84 (75-105)
6 hamsters, deficient diet low in fat	57	4.24 (3.90-4.67)		100 (96-109)

\* The number of animals in the average.

what greater, 19 per cent; the wide range of values for these vitamin E-deficient animals is the result of the great variability in the onset of dystrophy and the lack of definite criteria for judging how far it has progressed. In the rabbits the lowest creatine values appear to be associated with the final stages of a dystrophy developing slowly over a long period of time and are perhaps due to extensive replacement of active tissue by scar tissue.

The changes in chloride concentration are greater than those in creatine and in all cases the ranges are smaller with respect to the total change.

In the rabbits the figure is 280 per cent of the normal, in the grown rats 140 per cent, and in the hamsters 150 to 180 per cent of normal.

Each of these alterations from normal conditions may be the expression of some specific departure from the usual course of metabolic events and since the variations are not parallel the events concerned would appear to be sequential rather than synchronous. The lack of vitamin E produces a primary response, perhaps not included in these measurements, and the other responses follow in the train of the first; the primary response appears to be the altered rate of oxidation.

The cure of nutritional muscular dystrophy by administration of  $\alpha$ -tocopherol has been reported by many investigators. Individual rabbits

TABLE II  
*Influence of Single Dose of  $\alpha$ -Tocopherol on  $QO_2$  of Vitamin E-Deficient Hamster Muscle*

Diet	Weight	Interval after dose (1 mg.)	$QO_2$	Chlorides
	gm.		c.mm. $O_2$	mg. per cent
Low fat	60	35 hrs.	1.79	79
	64	33.5 hrs.	1.90	92
	50	32.5 "	1.84	74
	63	27.5 "	1.63	75
	53	132 "	2.25	112
	93*	Normal diet	1.70	57
	57*	No treatment	4.24	100
High fat	63	48 hrs.	1.79	83
	63	30 "	1.81	81
	67	12 days	1.91	69
	93*	Normal diet	1.70	57
	74*	No treatment	3.02	84

\* Average of several animals.

on a deficient diet have been cured repeatedly with final complete restoration of normal muscle histology (21). Whether and how soon after the giving of tocopherol all of the altered properties become normal, and in what order they are restored, are important questions.

Provisional answers were first sought in experiments on demonstrably dystrophic hamsters. At varying intervals after administration of 1 mg. of  $\alpha$ -tocopherol their muscles were examined for oxygen consumption and chloride content and, as Table II indicates, the former had fallen to normal levels in as short a time as 27 hours, whereas the chloride content had declined somewhat but was still considerably above normal. In one animal, 132 hours after the curative dose, both figures were again high; in another they were only a little above normal after 12 days. Since the

actual levels at the time of tocopherol feeding were not known, biopsy experiments on a larger animal were indicated.

An initial muscle sample was removed aseptically from the leg of a rabbit under ether and pentothal anesthesia. After recovery from the anesthesia, the rabbit was given  $\alpha$ -tocopherol acetate by mouth and at varying intervals thereafter a muscle sample was obtained from the other hind leg.

The results of these more direct experiments (Table III) demonstrated a significant diminution of oxygen consumption due to the tocopherol feeding, 28 to 43 per cent in 31 to 48 hours, and 34 per cent after as short a period as 10 hours.

TABLE III

*Effect of Oral Administration of  $\alpha$ -Tocopherol Acetate on Oxygen Consumption and Creatine Content of Dystrophic Rabbit Muscle; Biopsy Experiments*

Animal No.	Initial		Amount given mg.	After administration of $\alpha$ -tocopherol acetate				
	O <sub>2</sub>	Creatine		Interval	O <sub>2</sub>	Decrease	Creatine	Chloride
	c.mm. O <sub>2</sub>	mg. per cent		hrs.	c.mm. O <sub>2</sub>	per cent	mg. per cent	mg. per cent
4	2.46		25	48	1.75	28	460	79
2	3.64	465	50	48	2.08	43	431	126
9	2.25	505	25	48	1.43	36	445	105
13	2.87	492	25	42	1.63	43	490	
6	3.18	298	30	31	1.95	39	360	137
14	3.18	308	20	10	2.00	34	408	
3 normal rabbits (average) . . . . .	1.36	498		48	1.56		471	51

The original creatine figures were not greatly below normal and the tocopherol feeding produced no significant change. The high chloride content of the second muscle samples indicates that the degenerated state still existed. A single experiment in which the chloride content was determined in both samples showed that no change had taken place within 48 hours.

Neither the operation nor the anesthesia produced any change in the oxygen uptake or creatine content of muscles from normal animals (Table III). The chloride content after the second operation was normal.

These data indicate that the return toward a normal rate of oxidation in muscle is the primary result of the feeding of tocopherol and that the other changes produced are secondary and consequent upon a restoration of normal oxidative processes. The action of the  $\alpha$ -tocopherol was fairly rapid. The time required for absorption and transport of the  $\alpha$ -tocopherol

acetate may be the main limiting factor in the speed with which its effect on the processes of oxidation in dystrophic muscle is manifested.

#### SUMMARY

Dystrophic muscle from vitamin E-deficient animals as compared with muscle from normals has a much higher oxygen consumption, a somewhat lower creatine content, and a higher chloride content. Dystrophic hamster muscle showed the most striking increase in oxygen consumption, 250 per cent of the normal. Paralyzed nursing rats had a  $Q_{O_2}$  160 per cent of the normal. Oxygen uptake appeared to increase with the severity of the dystrophic condition. The creatine content was variably decreased but not greatly except in slowly developing, severe dystrophy.

Oral administration of  $\alpha$ -tocopherol to the dystrophic hamster resulted in the lowering of oxygen consumption toward the normal within 27 hours. The chloride content remained high even after 48 hours.

Biopsy experiments on the rabbit demonstrated conclusively that  $\alpha$ -tocopherol acetate given by mouth lowered the high initial oxygen consumption of the muscle toward normal and in as short a time as 10 hours. The concentration of creatine was not significantly altered and the chloride content remained high. Neither the operation nor the anesthesia had any effect on the oxygen consumption or creatine content of muscle from normal animals.

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# THE INFLUENCE OF PARENTERAL ADMINISTRATION OF $\alpha$ -TOCOPHEROL PHOSPHATE ON THE METABOLIC PROCESSES IN DYSTROPHIC MUSCLE

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The biopsy experiments described in the preceding paper (1) demonstrated that a reduction in the oxygen uptake of dystrophic rabbit muscle followed the oral administration of  $\alpha$ -tocopherol acetate in as short a time as 10 hours. A part of this interval was required for alimentary absorption of the tocopherol, a part for its distribution and diffusion into the muscle cells, and the remainder for the initiation of the processes which altered the metabolism. For an understanding of the rôle of tocopherol it was obviously desirable to rule out the first and as much of the second part of this interval as possible. Parenteral administration of tocopherol acetate or of tocopherol itself is even less efficient than oral (2, 3) and intravenous injection of either seemed ill advised. Tocopherol phosphate, on the other hand, is somewhat soluble in water and has been shown to be effective when given by injection (4). A series of multiple biopsy experiments was therefore planned, in which a slow intravenous injection of  $\alpha$ -tocopherol phosphate via<sup>1</sup> ear vein should immediately follow the removal of the first muscle sample from dystrophic rabbits. Other portions of muscle were removed at suitable intervals thereafter.

Divided doses of amytal which kept the animals in constant narcosis were supplemented with ether anesthesia, when necessary, during the dissection of muscle. Hemorrhage was readily controlled by careful dissection and the use of thrombin solution.<sup>2</sup> Usually two portions of semitendinosus muscle were taken from each hind leg for the determination of oxygen uptake and creatine content, and with the last sample, a larger portion, from the gastrocnemius, for chloride estimation. The animals were kept warm, the circulation was well maintained, and the blood supply to the remaining muscles was not disturbed.

## DISCUSSION

As seen in Fig. 1, the high oxygen consumption of dystrophic muscle was reduced by an average of 33 per cent during the 1st hour after intravenous administration of  $\alpha$ -tocopherol phosphate; by the end of the 4th

<sup>1</sup> Kindly supplied by Hoffmann-La Roche, Inc.

<sup>2</sup> Kindness of Dr. W. H. Seegers.



hour the average reduction was 49 per cent, well along toward normal figures, with a satisfactorily narrow range of 47 to 52 per cent. The most pronounced effect occurred within the 1st hour, after which the return toward a normal metabolic state proceeded at a more gradual rate.

Accompanying the decrease in oxygen consumption of the muscle, there was a drastic reduction in creatine content (Fig. 1). 1 hour after the injection the creatine content was only one-half the initial and paralleled the rapid decline in oxygen consumption. No greater decrease was observed when the interval was 2 hours. In all cases, the creatine content

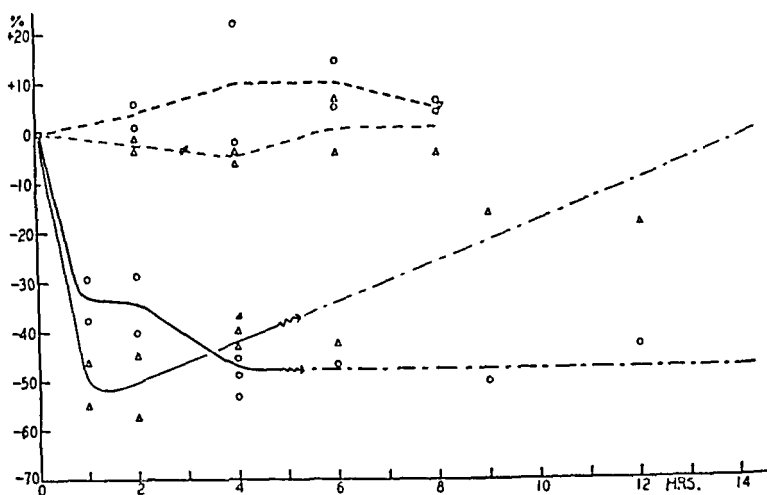


FIG. 1. Percentage change from the initial oxygen consumption and creatine content of dystrophic and normal muscle induced by the parenteral administration of  $\alpha$ -tocopherol phosphate. O, oxygen consumption;  $\Delta$ , creatine; the heavy and thin solid lines suggest the course of changes in oxygen consumption and muscle creatine in dystrophic rabbit muscle, the probable later course being indicated by the projected broken lines; the heavy and thin broken lines at the top indicate the course of oxygen consumption and creatine content of normal rabbit muscle; one normal animal was injected with  $\alpha$ -tocopherol phosphate.

had begun to rise by the 4th hour but was still 40 per cent below the initial value at that time and showed a progressive rise with time.<sup>3</sup> The chloride content of the muscle taken at the end of the experiment was still high and somewhat proportional to the original high oxygen consumption.

The biopsy operation itself had no effect on the oxygen consumption or the creatine content of muscle of a normal rabbit over as long a period as 8 hours (Fig. 1); the creatine content did increase slightly after 6 and 8

<sup>3</sup> One experiment indicated that the injection of  $\alpha$ -tocopherol phosphate progressively lowered the creatine excretion of a dystrophic rabbit to correspond in respect to time with the subsequent rise in muscle creatine content.

hours. Likewise there was no effect when  $\alpha$ -tocopherol phosphate was injected into a normal animal except for a possible slight *increase* in the oxygen consumption after 4 and 6 hours with a return to the initial level at the 8th hour. This increase, if significant, makes the response of vitamin E-deficient animals to  $\alpha$ -tocopherol phosphate even more striking. The normal creatine content was not altered, and the chloride content at the end of 8 hours was normal in both cases.

The decrease in oxygen consumption and creatine content of muscle of dystrophic rabbits following the intravenous injection of  $\alpha$ -tocopherol phosphate is thus not an artifact produced by shock, anoxia, or as the result of mechanical, circulatory, or other disturbances. The rapidity of the response is due to the immediate availability of  $\alpha$ -tocopherol phosphate, which is lacking in dystrophic muscle.

The prompt lowering of the rate of oxidation by  $\alpha$ -tocopherol phosphate in these biopsy experiments is in keeping with the known effect of tocopherol in dystrophic animals and suggests that the process of restoration, so far as oxygen uptake is concerned, is probably complete within little more than 4 hours. The lowering of the creatine content during this period is not consistent with the end-result of tocopherol treatment of dystrophic animals. This result, ultimately, is to increase the creatine content to normal. The initial decrease may be tentatively explained on the basis of an immediate decline in the rate of formation of creatine without a simultaneous decline in the rate of its loss by muscle tissue. The creatine turnover in a dystrophic rabbit must be considerable. In a well developed case the daily creatine excretion in the urine may account for as much as 20 per cent of the entire creatine stores in the animal. We would interpret the changes in muscle creatine to indicate a continued loss of creatine from muscle tissue with a diminished rate of formation during the 1st and 2nd hours following the giving of  $\alpha$ -tocopherol phosphate. Thereafter the rate of loss also begins to decline with the result that creatine can begin to accumulate and continues to do so until normal levels are obtained. The equilibrium between rate of formation and rate of loss may be reached in as short a time as 10 hours but the interval is usually much longer ((1) Table III). The interval required for restoration of normal chloride content is still longer. However the oxygen uptake and creatine metabolism may be related to tocopherol phosphate, it is evident that this substance is intimately connected with both processes; it may be a member of the complicated and yet unknown enzyme systems by which the physiological integrity of muscle tissue is maintained.

#### SUMMARY

Biopsy experiments on rabbits have shown that parenteral administration of  $\alpha$ -tocopherol phosphate caused the high oxygen consumption of

dystrophic rabbit muscle to drop 34 per cent within the 1st hour; the fall continued during 4 hours to 49 per cent, a decrease to nearly normal figures. A sharp decline in muscle creatine content accompanied the lowering of oxygen consumption during the first 2 hours; at 4 hours there was a slight rise but the figures were still far below the initial, already low, dystrophic level. This result is the opposite of the final effect of tocopherol on the muscle creatine of dystrophic rabbits when they are cured. The chloride concentration of the muscle remained high throughout and was not immediately influenced by tocopherol.

The biopsy operation itself had no effect on either oxygen uptake or creatine content of normal muscle.  $\alpha$ -Tocopherol phosphate had little, if any, effect on normal muscle; the only effect was a slight increase in oxygen consumption after 4 and 6 hours.

The data are interpreted to mean that  $\alpha$ -tocopherol phosphate is intimately connected with the complicated and yet unknown enzyme systems by which the physiological integrity of muscle tissue is maintained.

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# THE IN VITRO EFFECT OF $\alpha$ -TOCOPHEROL AND ITS PHOSPHATE DERIVATIVE ON OXIDATION IN MUSCLE TISSUE\*

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The biopsy experiments on dystrophic rabbits, described in an earlier paper (2), showed that the administration of  $\alpha$ -tocopherol phosphate to a dystrophic rabbit lowers the excessive metabolic rate of muscle tissue to a normal level within the relatively short interval of 4 hours. The restoration of normal creatine appears to follow next, and of chloride only after a much longer, and as yet undetermined, interval. Obviously,  $\alpha$ -tocopherol plays a rôle in the regulation of normal muscle processes. The following experiments establish the fact that  $\alpha$ -tocopherol phosphate has a direct action on muscle cells *in vitro* and indicate that it affects the succinoxidase system.

Muscles from rabbits and hamsters and three vitamin E-deficient rats were studied. The control medium for the measurement of oxygen consumption of tissue slices was a modified Locke's solution; the experimental medium was this same solution containing 5 mg. per cent of  $\alpha$ -tocopherol phosphate or of  $\alpha$ -tocopherol as an emulsion. The former was prepared by dissolving 5 mg. of the phosphate in a few drops of water and making up to 100 cc. with the Locke's solution. Parallel duplicate (or more) measurements of oxygen uptake of all muscles were made simultaneously in both control and experimental nutrient solutions.

As seen in Table I, the oxygen consumption of muscle from vitamin E-deficient rabbits and hamsters in the medium containing  $\alpha$ -tocopherol phosphate averaged 41 and 36 per cent less than that of slices from the same muscle in the unsupplemented medium. The presence of  $\alpha$ -tocopherol phosphate reduced the high oxygen consumption nearly to the normal rate. The  $Q_{O_2}$  of the vitamin E-deficient rat muscle was not significantly altered, but the oxygen consumption had not yet increased greatly beyond the normal.

The oxygen consumption of the muscle of normal animals was not altered by the medium containing  $\alpha$ -tocopherol phosphate. When the oxygen consumption had already been lowered toward normal by previous administration of  $\alpha$ -tocopherol acetate to dystrophic rabbits, the addition of

\* A preliminary report of some of these results has been published (1).

$\alpha$ -tocopherol phosphate to the medium caused no significant change. The  $Q_{O_2}$  of muscle slices after immersion for 3 minutes in boiling Locke's solution was negligible and was not influenced by  $\alpha$ -tocopherol phosphate. The  $\alpha$ -tocopherol phosphate thus participates in a biological system and does not merely act as a non-biological antioxidant.

Results with  $\alpha$ -tocopherol *in vitro* were inconclusive (Table II). Four experiments on the dystrophic hamster muscle showed no significant change and a large variability. Muscle from normal hamsters showed no change. Whether the emulsion secured was not sufficiently fine, whether absorption

TABLE I

*In Vitro Influence of  $\alpha$ -Tocopherol Phosphate on Oxygen Uptake of Muscle from Vitamin E-Deprived and Normal Animals*

Animal and condition	No. of animals	$Q_{O_2}$ , average and range		Decrease	Tissue creatine
		Control Locke's solution	Experimental Locke's solution containing 5 mg. per cent $\alpha$ -tocopherol phosphate		
		<i>c.mm. O<sub>2</sub></i>	<i>c.mm. O<sub>2</sub></i>	<i>per cent</i>	<i>mg. per cent</i>
Vitamin E-deficient rabbit	4	2.54 (1.75-3.23)	1.45 (1.20-1.64)	41 (26-49)	457 (423-515)
“ “ hamster	5	2.89 (2.31-3.35)	1.86 (1.61-2.05)	36 (30-39)	271 (213-329)
Destroyed*	3	0.06 (0.04-0.1)	0.08 (0.04-0.12)		
Vitamin E-deficient rat	3	1.73 (1.32-2.25)	1.59 (1.24-2.10)	8 (6-12)	446 (431-460)
Normal rabbit	2	1.34 (1.30-1.39)	1.40 (1.30-1.51)	-4 (0 to -9)	454 (439-468)
Vitamin E-deficient rabbit, treated†	2	1.72 (1.65-1.80)	1.62 (1.58-1.65)	6 (4-8)	495 (490-501)
Normal hamster	1	1.74	1.78	-2	357

\* The tissue slices were dropped in boiling Locke's solution and heated 3 minutes

† Given a therapeutic dose of 25 mg. of  $\alpha$ -tocopherol acetate 48 hours previously.

into the cell was unsatisfactory, or whether  $\alpha$ -tocopherol requires phosphorylation to be active are as yet open questions.

Since  $\alpha$ -tocopherol phosphate did not affect the  $Q_{O_2}$  of normal muscle or of muscle from dystrophic animals that had previously been given  $\alpha$ -tocopherol, it may be assumed that these muscles already contained the optimum amounts of tocopherol necessary for normal metabolic processes. Some form of  $\alpha$ -tocopherol is thus directly concerned in the enzyme systems of muscle tissue to inhibit or at least regulate some step or steps in the oxidation-reduction systems. For further insight into the nature of these sys-

terms in which  $\alpha$ -tocopherol phosphate seems to participate, comparisons were made of the oxygen consumption of normal and vitamin E-deficient muscle when sliced, minced, and homogenized.

The thigh muscle from normal and vitamin E-deprived hamsters was used. Minced tissue was prepared by the tissue mincer of Seevers and Shideman (3). Slices and portions of the mince were rapidly weighed in wet condition with a precision balance. The homogenate was prepared by homogenizing (4) a weighed amount of mince in the nutrient solution. After appropriate dilution, a measured amount was pipetted into the respiration vessels. Microscopic examination revealed that few if any intact cells remained. A like sample of muscle was weighed and dried at  $110^\circ$ , from which the dry weight of wet tissue used was calculated.

TABLE II

*In Vitro Effect of  $\alpha$ -Tocopherol on Oxygen Uptake of Muscle from Vitamin E-Deprived and Normal Hamsters*

Condition of animal	$QO_2$ for muscle		Decrease	Creatine
	Control Locke's solution	Experimental Locke's solution containing 5 mg. per cent $\alpha$ -tocopherol		
	<i>c.mm. O<sub>2</sub></i>	<i>c.mm. O<sub>2</sub></i>	<i>per cent</i>	<i>mg. per cent</i>
Normal.....	1.50	1.65	-10	357
".....	1.68	1.70	0	387
Vitamin E-deprived.	2.73	1.98	27	351
Vitamin E-deprived	2.13	2.88	-34	200
Vitamin E-deprived.	2.43	2.49	0	331
Vitamin E-deprived..	2.85	2.74	4	324

Mincing and homogenizing lowered the  $QO_2$  of normal muscle by 54 and 60 per cent respectively and lowered the  $QO_2$  of dystrophic muscle 71 and 78 per cent respectively (Table III). Although the  $QO_2$  of the dystrophic muscle slice was 61 per cent higher than the  $QO_2$  of normal muscle slice, the muscle mince and homogenate gave virtually identical oxygen consumption values for both normal and dystrophic muscle. The range of values for the mince was the same for both types of muscle, but with the homogenates the dystrophic muscle had a greater range than the normal. The amount of homogenate or mince used had no significant influence on the trend of the oxygen uptake.

The high oxygen uptake of vitamin E-deficient muscle is thus dependent on an intact structure. It is unlikely that the cell wall controls the diffusion of oxygen or that the small amount of  $\alpha$ -tocopherol phosphate necessary to cause a reduction in oxygen consumption would alter the state of

the cell wall or block the diffusion of oxygen. A more plausible alternative is that an enzyme system is involved that depends, for its complete function, on the cell structure to keep its components intact. Such an enzyme system should show the dilution effect, in that it has one or more water-soluble components that would diffuse away from the rest of the system and thus leave too low a concentration for proper function.

One of the systems that demonstrates this dilution effect is the succinoxidase system. The soluble, diffusible factor in this system is cytochrome *c* (5). A study of this system in the vitamin E-deficient tissue was

TABLE III

*Effect of Mincing and Homogenization on Oxygen Uptake of Normal and Vitamin E-Deprived Hamster Muscle*

Condition	Slice	Mince		Homogenate	
	QO <sub>2</sub>	Amount (dry basis)	QO <sub>2</sub>	Amount (dry basis)	QO <sub>2</sub>
	<i>c.mm.O<sub>2</sub></i>	<i>mg.</i>	<i>c.mm. O<sub>2</sub></i>	<i>mg.</i>	<i>c.mm. O<sub>2</sub></i>
Normal	1.85	25.9	1.08		
	2.17	26.4	0.75	27.6	0.87
	1.87	42.0	0.47	38.0	0.70
	1.57	30.4	0.71	30.6	0.65
	1.44	29.4	0.92	24.9	0.57
	1.36			40.3	0.59
Average .....	1.71	30.8	0.79	32.3	0.68
Vitamin E - deprived	3.46	20.5	0.99	17.2	1.10
	2.78	15.8	1.06	11.0	0.23
	2.38	24.1	0.63	22.8	0.44
	2.90	28.4	0.78	27.6	0.79
	2.25	26.0	0.57		
Average.....	2.75	22.9	0.81	19.6	0.64

undertaken because of the simplicity of the method of isolating the system and eliminating side reactions and because the cytochrome *c*-cytochrome oxidase components involved are believed to be a primary point of entry for oxygen into biological oxidations.

Succinoxidase activity was determined by the method of Potter (5) on the muscles of normal and dystrophic hamsters, and of normal and paralyzed suckling rats. The sodium succinate solution was prepared so that 0.1 cc. gave a concentration of 0.025 mole in the final (3 cc.) reaction mixture. The phosphate buffer, 0.03 M, was adjusted to pH 7.4 and contained 20  $\gamma$  per cent of calcium. Cytochrome *c* was prepared by the

method of Keilin and Hartree (6). The tissue was minced and homogenized in a known amount of cold phosphate buffer, and measured out with a calibrated pipette. After the control samples were removed,  $\alpha$ -tocopherol phosphate was added to the homogenate in the form of a water solution, and, to secure uniform mixing, this was homogenized again for about 1 minute.

$\alpha$ -Tocopherol was added as such to the homogenate and the mixture was also again homogenized. On occasion, desoxycholic acid was added in advance of the  $\alpha$ -tocopherol. Each vessel contained the tissue homogenate with or without additions, a cytochrome *c* preparation providing approximately  $2$  to  $3 \times 10^{-8}$  mole,  $0.025$  mole of succinate, and buffer to make  $3.0$  cc. The center well contained filter paper moistened with KOH solution. The succinate and additional buffer were placed in the side

TABLE IV

*Vitamin E Deprivation and Succinoxidase Activity of Hamster and Rat Muscle*

Animal and condition	No. of animals	$Q_{O_2}$ , succinoxidase activity		Per cent above normal*	$Q_{O_2}$ of muscle slice
		1st period	Average for 3 periods		
		<i>c.mm. O<sub>2</sub></i>	<i>c.mm. O<sub>2</sub></i>		<i>c.mm. O<sub>2</sub></i>
Normal hamster	4	15.8 (14.9-16.4)	14.9 (13.9-16.4)		1.75 (3)† (1.70-1.81)
" suckling rat	2	14.9 (13.7-16.1)	15.0 (14.0-16.0)		1.88 (1.77-1.98)
Dystrophic hamster	12	42.1 (25.2-61.7)	38.4 (24.9-60.0)	162 (63-295)	3.61 (4)† (3.00-4.11)
Paralytic suckling rat	6	18.9 (17.5-20.0)	18.3 (17.4-19.5)	24 (13-32)	3.33 (2)† (2.80-3.86)

\* Based on the first 10 minutes.

† The number of animals in the average.

arm and tipped into the reaction chamber after the flasks had been equilibrated at  $38^\circ$  for 15 minutes. 3 minutes later, the first reading was made, and subsequent readings followed at intervals of 5 or 10 minutes. The results were calculated in terms of  $Q_{O_2}$  on the basis of the 10 minute readings. Parallel determinations of the  $Q_{O_2}$  of muscle slices were also made.

The succinoxidase activity of dystrophic hamster muscle was much (162 per cent) above that of normal muscle (Table IV). The difference between normal and paralyzed suckling rats was much less. The succinoxidase activity of normal hamster muscles in terms of  $Q_{O_2}$  was  $14.9$  to  $16.4$  c.mm. of  $O_2$ , values somewhat lower than those reported by Axelrod *et al.* (7) for the thigh muscle of the rat. As a routine procedure in these experiments the succinoxidase activity of each tissue was determined at



levels of approximately 2, 3, and  $5 \times 10^{-8}$  mole of added cytochrome *c*. These values were constant enough in every determination to serve as checks upon each other and to indicate true succinoxidase activity. Dystrophic hamster muscle had a  $Q_{O_2}$  value for succinoxidase activity of 42.1 c.mm. of  $O_2$ . The range was considerable, 25.2 to 61.7 c.mm. of  $O_2$ , and appeared to be related to the condition of dystrophy; the more severely dystrophic, as judged externally, the higher was the muscle succinoxidase activity. In no case was there an overlap in values for dystrophic and normal muscles. The succinoxidase activity declined only slightly throughout the 30 minute period.

Proof that the oxygen uptake measured was due to the succinoxidase system was secured by the use of malonate. The addition of 0.03 mole of

TABLE V

*Effect of  $\alpha$ -Tocopherol Phosphate on Succinoxidase System of Vitamin E-Deprived Hamster Muscle*

Preparation No.	$Q_{O_2}$ , succinoxidase activity without $\alpha$ -tocopherol phosphate	$\alpha$ -Tocopherol phosphate present	$Q_{O_2}$ , succinoxidase activity in presence of $\alpha$ -tocopherol phosphate	Decrease
	c.mm. $O_2$	mg. per cent	c.mm. $O_2$	per cent
1	54.0	5	21.8	60
2	54.0	5	21.8	60
3	61.7	20	12.0	80
4	49.6	2	38.0	23
5	47.0	5	23.7	50
6	54.6	10	5.0	91
7	54.0	5	15.7	71
8	26.9	3	16.6	38
Average.....	50.3		19.3	59

sodium malonate to the system blocked 87 per cent of the oxygen uptake. Preparations from muscles of these deficient hamsters gave an average  $Q_{O_2}$  of 28.3 c.mm. of  $O_2$  (21.8 to 32.0); in the presence of malonate the figure was 3.82 (1.73 to 4.66).

If the succinoxidase system is the one upon which the increase in oxygen consumption of dystrophic muscle depends, then,  $\alpha$ -tocopherol phosphate should lower it just as it lowered the  $Q_{O_2}$  of dystrophic muscle slices. The following observations show that this is actually the case.

As seen in Table V,  $\alpha$ -tocopherol phosphate diminished the high succinoxidase activity in vitamin E-deficient hamster muscle by 59 per cent (23 to 91 per cent); the amount of reduction was somewhat proportional to the concentration of  $\alpha$ -tocopherol phosphate present. The succinoxidase activity was lowered nearly to the normal in all but two extreme cases.

The small amount of  $\alpha$ -tocopherol phosphate (0.06 to 0.6 mg. per flask) could hardly be effective in any other way than as a chemical regulator of the succinoxidase system. The decreased activity of this system agreed well with the reduced oxygen consumption of dystrophic muscle slices effected by  $\alpha$ -tocopherol phosphate *in vitro* and *in vivo*.

$\alpha$ -Tocopherol did not lower the oxygen consumption of dystrophic muscle slices *in vitro*. The inability of the muscle cell to absorb this water-insoluble material, or the lack of a sufficiently fine emulsion, might be limiting factors. Since the succinoxidase activity was measured on homogenates, the first postulate was excluded and the second could be tested by the use of some substance that lowered surface tension, such as desoxycholic acid.

$\alpha$ -Tocopherol had no effect on the succinoxidase activity of vitamin E-deficient muscle. Desoxycholic acid did not alter the  $Q_{O_2}$ , nor did it influence  $\alpha$ -tocopherol in altering the succinoxidase activity of dystrophic muscle.  $\alpha$ -Tocopherol as such, therefore, appears to have no direct influence on the enzyme system but must be phosphorylated or converted into some other water-soluble form to be effective.

#### DISCUSSION

The increased succinoxidase activity of vitamin E-deficient muscle, and its subsequent reduction by *in vitro* addition of  $\alpha$ -tocopherol phosphate, have implications for biological oxidation in general. The fundamental position of the cytochromes in aerobic cellular respiration is obvious from the fact that aerobic respiration is largely blocked by cyanide (8), and that all carbohydrate oxidation is believed to take place through the cytochromes (9). Further, the succinate-succinic dehydrogenase system has long been recognized as one of the reducing systems for cytochrome. A final link for this and perhaps other systems has recently been proposed in the very labile flavoprotein, cytochrome reductase (10, 5). At the moment, the major components of the complete succinoxidase system would appear to be succinate, succinic dehydrogenase, cytochrome *c*, cytochrome reductase, or some analogous enzyme, and cytochrome oxidase.

Skeletal muscle is high both in cytochrome oxidase and cytochrome *c* (11), but its resting oxygen consumption is much smaller than that of organs containing half the quantities of these components. Two possible explanations may be suggested for the relatively low oxygen uptake of normal muscle: (a) in proportion to its cytochrome content, the amount of hydrogen brought to the cytochrome is relatively small owing to the limited quantity of some enzyme in the "anaerobic" part of the system, or (b) there is some further mechanism which regulates the cytochrome system itself.

In vitamin E deficiency, the  $Q_{O_2}$  of skeletal muscle rises toward values

that would put it in its proper order and place with respect to its cytochrome *c*-cytochrome oxidase content. A higher content of some enzyme component might explain this increase, except that administration of tocopherol to a dystrophic animal restores the oxygen consumption to normal too rapidly for any readjustment of enzyme content to take place; also,  $\alpha$ -tocopherol phosphate *in vitro* immediately lowers the high  $Q_{O_2}$  of dystrophic muscle slices to a normal value. The succinoxidase system of muscle is also much less active than that of various organs. In the absence of tocopherol the activity of this system in muscle is greatly intensified, and the *in vitro* addition of  $\alpha$ -tocopherol phosphate reduces it toward normal intensity. Yeast cells, which contain no  $\alpha$ -tocopherol, demonstrate a reduced oxygen consumption when  $\alpha$ -tocopherol phosphate is added to the medium.

The oxidation-reduction potential of tocopherol lies at 0.20 volt at pH 7 (12). This is just below the potential of cytochrome *c* and between the potentials of cytochrome *c* and the known flavoproteins and other enzyme factors.  $\alpha$ -Tocopherol might thus prevent reduction of cytochrome *c* by inhibiting the transfer of hydrogen (or electrons) at some particular link in the chain. Substituted phenols have been shown to inhibit the action of some flavoproteins (13), more specifically of cytochrome reductase (14), but not of cytochrome oxidase.

To complicate matters further, it appears that  $\alpha$ -tocopherol may have to be in its phosphorylated form, as is generally the case with the other vitamins that enter into biological oxidations. The mechanism of tocopherol action might therefore be one which involves its dephosphorylation.

At present, any conception of the action of tocopherol must remain highly speculative. In the form of its phosphate it acts as a brake on the oxidative mechanism primarily of skeletal muscle and in its absence these oxidative processes in muscle run riot. An altered creatine metabolism is one of the results of this disturbance.

#### SUMMARY

The high oxygen consumption of dystrophic rabbit and hamster muscle slices was lowered 40 per cent and toward normal by the addition of  $\alpha$ -tocopherol phosphate to the medium. There was no influence on normal muscle slices. The  $Q_{O_2}$  of muscle slices after immersion for 3 minutes in boiling water was negligible and was not altered by  $\alpha$ -tocopherol phosphate. Results with  $\alpha$ -tocopherol *in vitro* were inconclusive.

Mincing and homogenizing lowered the  $Q_{O_2}$  of both normal and dystrophic muscle. Although the  $Q_{O_2}$  of the dystrophic muscle slices was 60 per cent higher than the normal, the mince and homogenate gave figures

for  $Q_{O_2}$ , which were, respectively, identical for normal and dystrophic muscle.

The succinoxidase activity of dystrophic hamster muscle was 160 per cent above that of normal muscle and somewhat proportional to the degree of dystrophy. The difference between normal and paralyzed suckling rat muscle was much less. The high activity was malonate-sensitive.

$\alpha$ -Tocopherol phosphate reduced the succinoxidase activity of dystrophic muscle by 59 per cent and toward normal; this agreed with the decrease in  $Q_{O_2}$  of muscle slices produced by  $\alpha$ -tocopherol phosphate *in vitro* and *in vivo*.  $\alpha$ -Tocopherol, either alone or with desoxycholic acid, failed to influence the oxygen uptake due to succinoxidase activity.

The possible rôle of  $\alpha$ -tocopherol phosphate in the metabolism of skeletal muscle is briefly discussed.

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# STUDY OF THE DISTRIBUTION OF SODIUM PREGNANEDIOL GLUCURONIDATE BETWEEN *n*-BUTANOL AND URINE OF PREGNANT WOMEN, TOGETHER WITH ITS PRACTICAL APPLICATION

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The method recently described by us (Allen and Viegiver (1)) for the determination of sodium pregnanediol glucuronide is a modification of the procedure originally developed by Venning (3, 4), differing only in the means used for finally measuring the compound. In both methods *n*-butanol is utilized for the extraction of the compound from urine. It is necessary to extract the urine at least three times with butanol to secure essentially complete extraction from the urine, provided the volume of butanol used for each extraction is approximately one-third to one-quarter of the volume of urine. This necessarily takes considerable time, since the layers of the two solvents separate slowly. In general, one extraction can be made daily. Consequently, 3 days are required to extract the urine and the volume of butanol obtained is relatively large.

A technical difficulty in the proper collection and preservation of the urine also appeared in the course of the work. The urine must be extracted before any hydrolysis of the compound into pregnanediol and glucuronic acid has occurred, since the method is based upon the isolation of the conjugated compound. If hydrolysis occurs prior to or during extraction, the free pregnanediol is not measured and low results are obtained. Venning recommends refrigeration of the urine during collection, followed by prompt extraction. We have found this procedure satisfactory when it was followed, but we have found it inconvenient and often impossible to secure satisfactory specimens, both in the hospital and in the home, when this method of preservation was supposedly followed. We decided, therefore, to collect all specimens at room temperature in large bottles containing 300 to 500 cc. of butanol, instructing the nurse, or the subject, to transfer each specimen as soon as voided to the bottle, followed by shaking of the bottle. This method of collection has proved very satisfactory. There appears to be no deconjugation of that portion of the compound which is in the butanol phase. The compound, when dissolved in butanol, appears to be stable. Whether or not that portion of the compound which remains in the urine phase is adequately preserved in the conjugated form is not

so clear. It was decided, therefore, to study in detail the recovery in various extractions to see whether a single extraction of urine could be used, since with this scheme the samples could be collected and the first extraction made immediately. The determination of the amount of the compound present in the butanol phase could be postponed indefinitely and the samples remain in the bottles used for collection.

The distribution of a soluble substance between two relatively immiscible solvents follows well recognized laws. It is possible, therefore, to predict the amount of the substance remaining in one phase (the urine) from the amount known to be present in the other phase (the butanol) provided that the relative solubility of the substance (sodium pregnanediol glucuronidate) in the two solvents is known, and provided that the exact volumes of the two phases are known. For example, if the compound is 13 times as soluble in butanol as in urine, the concentration in the urine would be only one-thirteenth that of the concentration in the butanol but the relative amounts in the two phases would depend on the relative volumes of butanol and urine. It is quite obvious, of course, that sodium pregnanediol glucuronidate is much more soluble in butanol than in urine. If it were not, the methods now used for the extraction would be entirely unsatisfactory. Consequently, it seemed feasible to carry out studies to determine the relative solubility of the compound in the two solvents and thereby make it possible to calculate the total amount present from the amount obtained by a single extraction with butanol. If the results proved fairly uniform, the method could be shortened by several days and any concern over deconjugation of the compound during collection could be eliminated.

The formulae which apply to the various ramifications of the problem are given in a paper by Ralls, Jordan, and Doisy (2) and are developed from the basic formula for the distribution of a soluble substance between two immiscible solvents.

$$X_n = A \left( \frac{b}{K + b} \right)^n$$

in which

$A$  = total amount of the compound (sodium pregnanediol glucuronidate) present

$X_n$  = amount remaining in the urine after  $n$  extractions

$K = C_b/C_u$ , where  $C_b$  = concentration in the butanol;  $C_u$  = concentration in the urine

$b = V_u/V_b$ , where  $V_u$  = volume of the urine;  $V_b$  = volume of the butanol

$n$  = number of extractions

From a consideration of the above formula it should be possible to determine  $K$ , the distribution constant, and  $A$ , the total amount present, if two successive extractions are made with butanol and the amount of sodium pregnanediol glucuronidate present in each extraction is deter-

mined. The addition of a known amount of the compound to a urine which had been previously extracted with butanol to remove all of the compound originally present would, of course, make it possible to determine  $K$  from only one extraction because  $X$ , the amount remaining in the urine, would necessarily be the difference between the amount added and the amount present in the first butanol extraction. In such an experiment all factors but  $K$  would be known. This plan was not followed out, however, because the extraction of urine with butanol also removes pigment and possibly other compounds which might alter the distribution of the compound, the experiment being, therefore, not an exact duplication of the procedure which would have to be followed in the case of the extraction of the compound from the original specimen of urine. It was decided, therefore, to obtain the data from analyses of pregnancy urines and consequently two factors in the formula would be unknown, namely  $K$  and  $A$ .  $X$ , of course, is known in terms of  $A$  because  $A - X =$  the amount extracted by the butanol.

An actual case will show that this is possible. In a given experiment 294 mg. were obtained in the first butanol phase and  $b = 2.94$ ; therefore,

$$A - 294 = A \left( \frac{2.94}{K + 2.94} \right)$$

In the second extraction 56 mg. were obtained in the butyl phase and  $b = 3.01$ ; therefore,

$$A - 294 - 56 = A \left( \frac{3.01}{K + 3.01} \right)$$

Solution of these two equations for  $A$  and  $K$ , gives  $K = 12.8$ ,  $A = 363$  mg.

The general formula used for the calculation of  $K$ , from the amount of compound present in the first two butanol fractions, can be derived from the original formula and is as follows:

$$K = \frac{Cb_1 Vu_1 - Cb_2 Vu_2}{Pb_2}$$

where  $Cb_1$  = the concentration in the first butanol fraction,  $Cb_2$  = the concentration in the second butanol fraction,  $Vu_1$  = the volume of the first urine phase,  $Vu_2$  = the volume of the second urine phase, and  $Pb_1$  and  $Pb_2$  = the amount of compound in the first and second butanol fractions, respectively.

The formula for the calculation of  $A$ , by use of the value of  $K$  obtained and the amount of compound in the first butanol fraction, is

$$A = \frac{Pb_1}{K} \left( K + \frac{Vu_1}{Vb_1} \right)$$



*Method*

Pooled urine from subjects in late pregnancy was divided into several equal volumes and approximately  $\frac{1}{3}$  volume of *n*-butanol (saturated with distilled water) was added to each. The solutions were thoroughly mixed by shaking and after standing overnight the two layers were separated by use of a siphon. The emulsion usually present at the urine-butanol interface was broken up by centrifugation, and the butanol and urine obtained added to the major portions of the two solutions. At this point, the total volumes of both the butanol and the urine were carefully measured and recorded.

Repeated extractions were carried out according to the above plan and the butanol phase recovered at the end of each extraction was treated as a separate unit. Consequently, at the completion of the several extractions of the urine, there were on hand for each original specimen of urine several solutions of butanol: one representing the first extraction, another the second extraction, etc. Because of the small amounts of sodium pregnanediol glucuronide anticipated in the third extraction, the butanol fractions from several aliquots were pooled in order that a sufficient amount of the compound might be present for analysis.

The determination of the sodium pregnanediol glucuronide present in the first, second, and third extractions with butanol was made by the titrimetric method previously described (1). The butanol fractions were distilled to dryness *in vacuo* (a water pump was used), and the residue was dissolved in 60 cc. of 0.1 *N* sodium hydroxide. The sodium hydroxide solution was extracted three times with 30 cc. volumes of butanol. The butanol was distilled to dryness *in vacuo*. The residue was dissolved in 10 cc. of water and the sodium pregnanediol glucuronide was precipitated by addition of 0.4 *M* lead nitrate. The precipitate was separated by centrifugation, the mother liquor discarded, and the complex extracted from the precipitate with 0.08 *N* sodium carbonate made up in 60 per cent alcohol. The amount of compound present in the sodium carbonate solution was then determined. The total amount obtained by separate determination of the amount in each of the three extractions should, of course, be the same as the total amount obtained by a single determination of the amount obtained by three extractions pooled before analysis. In each case controls were run by the method previously described.

The data obtained from the extraction of neutral urine (*i.e.*, urine to which no acid or base was added) are given in Table I. The volumes of the two phases and the amount of the compound in the butanol fractions of the various extractions are given. The concentration of the compound in the butanol phase was calculated and the values for  $Cb_1$ ,  $Vu_1$ ,  $Cb_2$ ,  $Vu_2$ , and  $Pb_2$  substituted in the formula and the value for  $K$  obtained for each separate observation. The values obtained for  $K$  vary between 8.4 and

16.5. The average values for the two groups of experiments, however, are virtually identical, 13.2 and 12.8. The variation is in reality only a manifestation in  $K$  of the relative error of the method.

The total amount present was obtained by totaling the amounts present in each of the three extractions and also by calculation from the amount present in the first extraction with the value 13 for  $K$ . The observed and calculated values are virtually identical. In the first group of experiments

TABLE I

*Amount of Sodium Pregnanediol Glucuronidate (NaPG) Obtained in Each of Three Extractions of Neutral Urine with Butanol*

Sample	1st extraction			2nd extraction			$K^*$	3rd extraction			Total in 3 extractions	Total calculated from 1st extraction, $K = 13, A$
	Urine volume, $V_{u1}$	Butanol volume, $V_{b1}$	NaPG in butanol, $Pb_1$	Urine volume, $V_{u2}$	Butanol volume, $V_{b2}$	NaPG in butanol, $Pb_2$		Urine volume, $V_{u3}$	Butanol volume, $V_{b3}$	NaPG in butanol, $Pb_3$		
	cc.	cc.	mg.	cc.	cc.	mg.		cc.	cc.	mg.	mg.	mg.
A	1350	250	109	1330	420	33	14.67	1320	445	10	152	154
B	1350	250	110	1340	425	38	12.48	1350	465	10	158	156
C	1350	250	105	1340	450	41	10.96	1345	460	11	157	149
D	1350	250	105	1340	435	32	14.64	1355	450	11	148	149
E	1350			Original method							159	
Average	1350	250	107	1337	432	36	13.19				155	152
F	1140	390	107	1140	380	16	16.51	1120	355	2.9	126	131
G	1140	375	103	1170	385	19	13.45	1130	375	2.9	125	127
H	1140	400	84	1160	385	21	8.39	1160	400	2.9	108	102
I	1140			Original method							124	
J	1140			" "							128	
Average	1140	388	98	1157	383	18.6	12.78				122	120

Samples A, B, C, D, and E are aliquots of one stock solution of pregnancy urine; Samples F, G, H, I, and J are aliquots of another stock solution of pregnancy urine.

$$* K = \frac{Cb_1 V_{u1} - Cb_2 V_{u2}}{Pb_2}$$

the averages were 155 mg. for the observed and 152 mg. for the calculated amount, and in the second group, 122 mg. for the observed and 120 mg. for the calculated. It is apparent, therefore, that the total amount present in the urine can be as accurately calculated from the portion of the total amount present in the first butanol phase as it can be determined by complete extraction. The shortened procedure does not sacrifice accuracy.

There is another step by which the procedure might also be simplified. The butanol-0.1 N sodium hydroxide distribution might be reduced to a

single extraction in a similar manner to the single extraction of the urine. This distribution was not studied in detail, however, because preliminary observations made when the method was being developed indicated that extraction was just as complete from urine made strongly alkaline before extraction with butanol as from neutral urine. Because of this, we suspected that the two steps could be combined; i.e., the urine could be made alkaline and a single extraction made with butanol, the butanol then being evaporated to dryness, the residue dissolved in water, and the sodium pregnanediol glucuronide precipitated directly with lead nitrate. This

TABLE II

*Amount of Sodium Pregnanediol Glucuronide (NaPG) Obtained in Each of Three Extractions of Alkalinized Urine with Butanol*

Sample	1st extraction			2nd extraction			K	3rd extraction			Total in 3 extractions	Total calculated from 1st extraction, K = 12
	Urine volume, $V_{u1}$	Butanol volume, $V_{b1}$	NaPG in butanol, $P_{b1}$	Urine volume, $V_{u2}$	Butanol volume, $V_{b2}$	NaPG in butanol, $P_{b2}$		Urine volume, $V_{u3}$	Butanol volume, $V_{b3}$	NaPG in butanol, $P_{b3}$		
	cc.	cc.	mg.	cc.	cc.	mg.		cc.	cc.	mg.	mg.	mg.
K	1310	290	108	1290	430	42	8.62	1322	452	6	156	149
L	1310	290	106	1275	435	31	12.51	1322	452	6	143	146
M	1310	285	106	1335	445	34	11.33	1322	452	6	146	146
N	Alkalinized urine, 3 extractions pooled; butanol-0.1 N NaOH distribution omitted										140	
O	Original method, 3 extractions pooled; butanol-0.1 N NaOH distribution included										138	
P	1260	370	83	1255	465	14.0	17.48				100*	106
Q	990	310	95	1015	350	18.0	13.96				117*	120
R	1330	370	55	1340	470	16.0	9.50				67*	71
Average.....							12.23					

Samples of K, L, M, N, and O are aliquots of one stock solution of pregnancy urine.

\* Values obtained by analysis of unalkalinized aliquots by the original method.

method was studied, therefore, to see whether the distribution constant, K, was the same for the butanol-alkaline urine as for the butanol-neutral urine phases.

The observations were made from experiments carried out by essentially the same procedure as those used for the determination of the K given above. Urines from various stages of pregnancy were pooled. A small amount of butanol was added, but not enough to produce a butanol layer, and the urine was kept in the refrigerator until several liters were obtained. Aliquots were taken from the stock solutions and approximately 28 to 30 cc. of 6 N sodium hydroxide were added to each 1000 cc. of urine. The

alkalinized urines were extracted three times with approximately  $\frac{1}{3}$  volume of butanol and the amount of sodium pregnanediol glucuronide was determined in the first and second butanol phases. The amount in the third phase was also determined but this was usually done by pooling the third butanol phase from three aliquots. The amount of the compound present in each phase was determined by the regular procedure, the distribution between 0.1 N sodium hydroxide and butanol being omitted. Control observations were made on aliquots by the original procedure.

The data obtained from the extraction of alkaline urine are given in Table II. The values obtained for  $K$  vary from 8.62 to 17.48, the average value being 12.2.

In view of the wide variation in the value for  $K$ , both in the first experiment with neutral urine and in the second experiment with alkaline urine, it seemed necessary to calculate the effect which these different values might have. If the value 8.0 is substituted in the formula, the calculated amount is 111 per cent of the correct amount (assuming  $K = 13$  to be correct), whereas if the value 18 is used for  $K$ , the calculated amount is 95 per cent of the correct amount, provided  $V_u/V_b = 3$ . The use of the average value, 13, therefore, seems justified. If the volume of butanol used were considerably smaller, so that the amount of the compound obtained in the first butanol phase was proportionally much less, error in the determination of the value of  $K$  would, of course, have a greater effect.

#### DISCUSSION

The information obtained from this study is directly applicable to any method now used, or which may be developed, for the extraction of sodium pregnanediol glucuronide from urine with butanol. If the Venning procedure is employed, a single extraction with butanol could be used, and the amount actually present could be readily calculated provided that the volumes of the butanol and the urine phases were noted. The result would doubtless be as accurate as if the urine had been more thoroughly extracted. The results of this study would not apply, however, to the extraction of pregnanediol itself from the urine after hydrolysis.

We have not attempted to analyze routine specimens by alkalinizing the urine before extraction with butanol, chiefly because the precipitate obtained with lead nitrate is rather bulky, but nevertheless manageable. Further experiments have revealed that the butanol-sodium hydroxide distribution apparently has to be retained in one form or another, because if it is omitted high results are frequently obtained. Also, when the butanol-sodium hydroxide distribution is omitted, the lead precipitate is usually rather gummy and the extraction with sodium carbonate may be unsatisfactory.

## SUMMARY

The distribution of sodium pregnanediol glucuronidate between butanol and urine has been studied. Sodium pregnanediol glucuronidate was found to be 13 times as soluble in butanol as it is in neutral urine and 12 times as soluble in butanol as it is in urine made strongly alkaline with sodium hydroxide. By the use of these values for the distribution constant,  $K$ , it is possible to calculate the total amount present in a given sample of urine from the amount actually obtained by a single extraction with approximately  $\frac{1}{3}$  volume of butanol. The values so obtained are virtually identical with the values obtained by complete extraction of the urine and considerable time and labor are saved.

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# DERIVATIVES OF TOBACCO MOSAIC VIRUS

## II. CARBOBENZOXY, *p*-CHLOROBENZOYL, AND BENZENESULFONYL VIRUS\*

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It was recently demonstrated that a considerable proportion of the amino and phenolic groupings of tobacco mosaic virus could be substituted with acetyl or phenylureido radicals without the loss of biological activity, and that the virus which was propagated in plants inoculated with the derivatives was normal virus (1). On the basis of this and additional evidence, it was concluded that the particular amino and phenolic groupings which were substituted were not important to the mechanism of reproduction of the virus. In order to confirm these observations and to determine whether this behavior was independent of the nature of the substituent groups, a number of other derivatives of the virus have been prepared.

Benzoylated tobacco mosaic virus possessing full biological activity has been described by Agatov (2). In the present investigation carbobenzoxy, *p*-chlorobenzoyl, and benzenesulfonyl derivatives of tobacco mosaic virus were studied. The derivatives were prepared by the addition of the appropriate acid chloride to a solution of virus buffered at pH 8. The reactions were, for the most part, carried out at room temperature and were allowed to proceed for different periods of time to yield derivatives of varying composition. The products were then tested for amino nitrogen, phenol plus indole groups, and specific virus activity.

The results are presented in Table I. The reagents are listed in the order of decreasing reactivity with the amino groups of the virus. The determinations of amino nitrogen by the Van Slyke method (3) are considered to be more reliable than those by the ninhydrin method (1). The maximum substitution of amino groups, namely 83 per cent, was comparable to that obtained in acetyl derivatives of the virus (1). The most extensive substitution of phenol plus indole groups was obtained in the benzenesulfonyl virus, in one preparation of which 28 per cent of these groups was affected. It may be noted that the benzenesulfonyl virus failed to exhibit a significant recovery of chromogenic power after treatment at pH 11. This finding was

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to be expected, however, on the basis of the known stability of the benzenesulfonyl-phenolic linkage to alkali (4). We have observed that the acetyl-phenolic linkage in acetylated virus undergoes spontaneous hydrolysis in aqueous solution (5). A similar breakdown in the carbobenzoxy, *p*-chlorobenzoyl, or benzenesulfonyl virus derivatives, if it occurs at all, takes place more slowly and, up to the present time, has been difficult to demonstrate conclusively.

The activities of the derivatives, as determined by tests on *Nicotiana glutinosa* L. (Table I), show that approximately 70 per cent of the amino groups of the virus could be substituted with carbobenzoxy or *p*-chlorobenzoyl groups without an appreciable inactivation of the virus, although further reaction in these cases was accompanied by a loss of activity. At

TABLE I

*Properties of Carbobenzoxy, p-Chlorobenzoyl, and Benzenesulfonyl Derivatives of Tobacco Mosaic Virus*

Reagent	Temperature	Time	NH <sub>2</sub> groups substituted		Phenol + indole groups substituted		Specific virus activity	
			Van Slyke	Nin-hydrin	pH 8	pH 11	<i>Nicotiana glutinosa</i>	<i>Phaseolus vulgaris</i>
	°C.	min.	per cent	per cent	per cent	per cent	per cent	per cent
Carbobenzoxy chloride	24	15	76	63	6	6	85	47
	24	30	77	72	6	3	67	14
	24	60	82	80	7	3	38	5
<i>p</i> -Chlorobenzoyl chloride	24	30	53	41	10	4	100	93
	24	60	69	59	11	5	98	51
	0	180	83	75	13	4	69	12
Benzenesulfonyl chloride	32	30	29	18	23	23	105	83
	32	60	53	37	28	29	65	35
	32	360	69	53	26	24	38	10

the same time, derivatives containing somewhat fewer benzenesulfonyl groups exhibited a diminished infectivity. Of the phenol plus indole groups, 20 per cent could be substituted without loss of biological activity on *N. glutinosa*. The results were thus far in close agreement with those obtained with the acetyl and phenylureido derivatives (1). They seemed to differ, however, when the measurements of activity were carried out on leaves of *Phaseolus vulgaris* L., as indicated in the last column of Table I. For example, in certain instances the activity was found to be appreciably lowered when it was determined on *P. vulgaris*, whereas it was essentially unchanged on *N. glutinosa*. Furthermore, the rate of inactivation measured on *P. vulgaris* was greater than that measured on *N. glutinosa*. Thus, in the extreme cases, the virus derivatives were 6 to 7 times as active on

*N. glutinosa* as on *P. vulgaris*. The reliability of the individual activity measurements was established by repeated determinations of the activities of some of the preparations on each of the test plants used. Since the virus derivatives exhibited different activities for the two hosts when the amino groups were substituted more than 50 to 70 per cent, it might be concluded that the differences in activity were the direct result of the specific chemical changes indicated. The possibility cannot be excluded, however, that the observed biological effects were the result of structural changes of a secondary nature not revealed by the chemical tests which were applied.

In the previous studies with acetyl and phenylureido derivatives of tobacco mosaic virus (1), the activity measurements were made more or less indiscriminately on plants of both *Phaseolus vulgaris* and *Nicotiana glutinosa*. No significant differences in activity on the two hosts were observed, but it may be recalled that only in a few cases were derivatives obtained in which losses in activity were manifested. In order to test for the possibility of a differential inactivation of the acetyl virus, the specific biological activity of partially inactivated acetyl virus was redetermined on both *P. vulgaris* and *N. glutinosa*. It was found that the preparation of virus which had been treated with ketene for four separate 1 hour periods (1) was approximately twice as active on *N. glutinosa* as on *P. vulgaris*. Since the acetyl derivative possessed a differential activity towards two hosts, it was concluded that this behavior, first discovered with the carbobenzoxy, *p*-chlorobenzoyl, and benzenesulfonyl derivatives of the virus, could not be attributed to the particular nature of the substituent groups in the latter cases.

It was next of interest to determine whether the inoculation of plants with the derivatives would give rise to the propagation of normal virus. Concentrations of  $10^{-4}$  gm. per cc. of the "30 minute" carbobenzoxy, the "60 minute" *p*-chlorobenzoyl, and the "360 minute" benzenesulfonyl virus preparations were rubbed on separate batches of young Turkish tobacco plants. The disease which was produced in each case was indistinguishable from the normal disease. After the diseased plants had grown for 3 weeks, the virus was isolated by differential centrifugation of the expressed juice. The purified virus preparations yielded by the ninhydrin and Herriott methods  $93 \pm 3$  and  $104 \pm 1$  per cent, respectively, of the color given by a sample of normal virus employed as a standard. The values obtained were within the limits of accuracy of the methods of analysis. The results, therefore, confirmed those obtained with the acetyl and phenylureido derivatives, and indicated that the particular amino and phenolic groupings which were substituted were unimportant to the mechanism of virus reproduction. From a consideration of the activity measurements as a whole, it is quite apparent that, within the limits of the variety of derivatives



studied, the *nature* of the chemical groups which were substituted on the amino and phenolic linkages of the virus had, *per se*, rather little specific effect on the physiological behavior of the derivatives.

An explanation of the observed biological infectivity of the various derivatives might reside in the ability of the plant cells to hydrolyze the artificially attached groupings and thereby to restore the virus to its normal form. Furthermore, the variation in specific virus activity on the two host plants studied may have been due to a difference in their ability to cleave the substituted radicals. Experiments were therefore carried out in an effort to answer the question of the ease of cleavage. Approximately 100 mg. samples of acetyl, phenylureido, carbobenzoxy, and *p*-chlorobenzoyl virus were each added to 50 gm. portions of mash obtained by grinding leaves of *Nicotiana glutinosa* in a meat grinder. The mixtures were allowed to stand for 24 hours at 25°, after which the virus samples were isolated and purified by the usual procedure. When tested for amino nitrogen, the isolated preparations, considered in the order given above, yielded values indicative of 70, 45, 78, and 47 per cent substitution. The values on the original preparations were 75, 50, 80, and 49 per cent, respectively. Similarly, no significant changes were observed in the values for phenol plus indole groups. The evidence suggests, therefore, that any enzymes which may have been present in the plant were unable to cleave the various groupings which had been attached to the protein by chemical means. It is possible, of course, that the conditions employed did not sufficiently simulate those existing within the plant cells.

It must finally be pointed out that most of the conclusions thus far made are dependent on the assumptions that in any given preparation of the derivatives the virus molecules were altered to approximately equal degrees and that no normal virus was present. Since a change in electrochemical properties was to be expected to accompany the alterations in chemical structure, a method of testing for homogeneity was provided by the use of the Tiselius electrophoresis apparatus. The measurements were made at pH 7.9 in veronal buffer in which 80 per cent of the ionic strength of 0.1 was provided by sodium chloride. The "30 minute" carbobenzoxy and the "60 minute" *p*-chlorobenzoyl derivatives each possessed a mobility of  $-8.7 \times 10^{-5}$  sq. cm. per volt second, and the "60 minute" benzenesulfonyl derivative a mobility of  $-8.3 \times 10^{-5}$  sq. cm. per volt second. Under the same conditions, the normal virus possessed a mobility of  $-8.0 \times 10^{-5}$  sq. cm. per volt second. Aside from the differences in mobility, the electrophoretic patterns given by the derivatives showed a degree of electrochemical homogeneity comparable to that shown by the normal virus. It was found that 5 per cent of normal virus, if present as a contaminant in preparations of the derivatives, could readily be detected by the present method. This

is illustrated by the scanning diagrams (6) presented in Fig. 1 for the normal virus, the carbobenzoxy virus, and a mixture containing 95 parts of carbobenzoxy virus and 5 parts of normal virus. From the appearance of the diagrams for each of the derivatives, it was concluded that the extent of contamination was less than 5 per cent. Electrophoresis diagrams obtained in experiments with equal proportions of normal virus with each of the derivatives were similar in character to those obtained with the corresponding mixtures in previous studies with the acetyl and phenylureido virus (1).

From an inspection of the activity measurements, as presented in Table I, it is apparent that the presence of even as much as 5 per cent of normal virus as an impurity could account for only a small proportion of the infec-

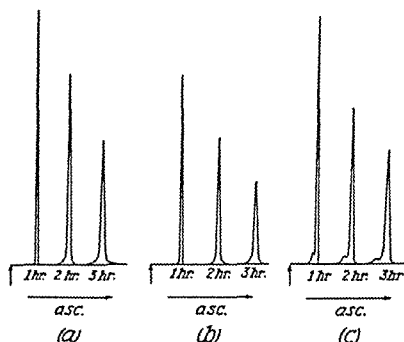


FIG. 1. Tracings of Longworth scanning diagrams of ascending boundaries obtained during electrophoresis of (a) 0.2 per cent normal virus, (b) 0.2 per cent carbobenzoxy virus, (c) a mixture of 0.2 per cent carbobenzoxy virus and 0.01 per cent normal virus. Field strength, approximately 3.2 volts per cm. The vertical arrows indicate starting positions of the boundaries.

tivity which was exhibited by most of the derivatives tested. It may safely be concluded, therefore, that individual units of the derivatives possessed biological activity. The possibility still remained, however, that a small amount of contamination with normal virus was responsible for the reproduction of normal virus observed in the Turkish tobacco plants which were infected with preparations of the derivatives. This question was considered in previous studies and, by special methods which were described, it was demonstrated that contamination could not have been responsible for the results which were obtained (1). Because of the almost complete agreement between the results of the present study and those of the earlier one, it was considered unnecessary to investigate this question further in the present case.

Although heritable virus derivatives have not yet been prepared *in vitro* by chemical means, virus derivatives have for the first time been prepared which exhibit different specific activities towards different hosts.

#### EXPERIMENTAL

*Preparation of Virus Derivatives*—A solution of 0.25 gm. of ultracentrifugally isolated tobacco mosaic virus in 25 cc. of 0.2 M phosphate buffer at pH 8.4 was treated with 0.2 cc. of the appropriate acid chloride with mechanical stirring. At the end of the chosen time, the excess of reagent and any insoluble material present were separated by means of centrifugation and the virus solution was dialyzed overnight against 40 liters of distilled water at 4° and used for the various chemical and biological studies.

*Analytical Methods*—The ninhydrin procedure was employed as described in a preceding paper (1). In the Van Slyke procedure it was found that the virus yielded slightly larger amounts of amino nitrogen at the higher temperatures encountered during the summer months. It was therefore necessary to control each determination by an analysis of normal virus at the same temperature.

The determinations of phenol plus indole groups were carried out by recent modifications of Herriott's methods in which sodium dodecyl sulfate in acid solution was employed in the pH 8 method to insure complete denaturation of the virus (5), and a period of 3 hours was allowed for the treatment with alkali in the pH 11 method to obtain essentially complete denaturation of the virus and saponification of the substituted phenolic linkages (4).

*Measurements of Specific Virus Activity*—The determinations of specific virus activity were carried out by the half leaf method as used in this laboratory (7). At least forty half leaves were employed for each measurement. An increased accuracy was obtained through the use of standard reference curves, which were obtained by determining the apparent activities of suitable dilutions of a standard virus solution when compared against the standard itself. Thus, dilutions of virus containing from  $0.025 \times 10^{-4}$  to  $0.4 \times 10^{-4}$  gm. per cc. were tested against the standard of  $0.5 \times 10^{-4}$  gm. per cc. The apparent per cent activities which were found were then plotted against the actual concentrations expressed as per cent of standard, as shown in Fig. 2. The resulting curves were employed as standards of reference for the determination of the actual activities of known concentrations of the virus derivatives after they in turn had been compared with the same standard used above. It is probable that these reference curves were strictly valid only for the set of conditions such as temperature, light, humidity, average number of lesions per half leaf, etc., under which they were determined. As further shown in Fig. 2, when

points on the standard reference curves were redetermined at later dates, fairly reproducible results were obtained in the case of the test plant, *Phaseolus vulgaris*. The results with *Nicotiana glutinosa* were, however, more erratic. The activity measurements for most of the virus derivatives reported in Table I were made within a few days of the determination of the standard curves, under very similar general conditions. In addition, the measurements of different preparations of any given derivative were usually all carried out at the same time, thus lending greater reliability to the differences in activities which were observed.

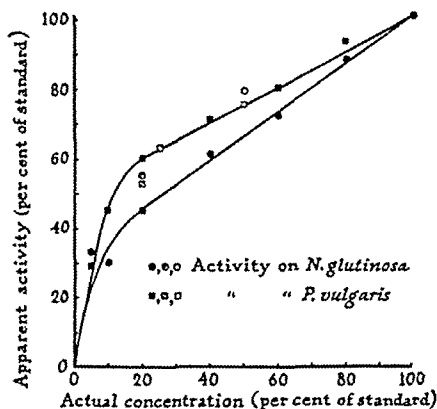


FIG. 2. Standard reference curves obtained by determining the apparent activities of dilutions of tobacco mosaic virus varying from  $0.025 \times 10^{-4}$  to  $0.4 \times 10^{-4}$  gm. per cc. when compared with a standard virus solution containing  $0.50 \times 10^{-4}$  gm. per cc. The solid symbols indicate the principal set of measurements on the basis of which the activities of the various derivatives were determined. The crossed and open symbols indicate additional measurements which were carried out later.

It was found that, when solutions of tobacco mosaic virus of unknown activity were so adjusted in concentration that they caused about the same average number of lesions per half leaf as did the standard virus solution against which they were compared, an accuracy of  $\pm 10$  per cent might be expected in activity measurements with test plants of *Phaseolus vulgaris*, and nearly as high an accuracy with test plants of *Nicotiana glutinosa*. These observations permitted us to check the accuracy of our data by a method illustrated by the following example. In duplicate measurements on leaves of *P. vulgaris*, the "180 minute" *p*-chlorobenzoyl preparation was found to possess an apparent specific virus activity of 46 to 48 per cent. These values, according to the appropriate standard reference curve, indicated an actual specific activity of only 11 per cent. The same prepara-

tion of virus derivative, when placed on test plants at a concentration 10 times that of the standard against which it was compared, gave an apparent activity of 106 per cent which, after correction on the standard reference curve and correction for the factor of concentration, yielded a final real activity of 11 per cent. By this same procedure, with concentrations of the virus derivative of 5 and of 8 times that of the standard, final real activities of 15 and 11 per cent, respectively, were obtained. An average value of 12 per cent was therefore quite well established. An analogous set of experiments with *N. glutinosa* as the test plant established the specific virus activity on this host as around 69 per cent. This preparation of virus therefore possessed approximately 6 times as much activity on the host plant, *N. glutinosa*, as on *P. vulgaris*. In view of these results, there is no reason to suspect any substantial error in the values given in Table I for the specific virus activities of the other preparations of virus derivatives.

#### SUMMARY

Carbobenzoxy, *p*-chlorobenzoyl, and benzenesulfonyl derivatives of tobacco mosaic virus have been prepared and demonstrated by means of electrophoretic measurements to contain less than 5 per cent of unaltered virus as a contaminant.

Measurements of specific virus activity on leaves of *Nicotiana glutinosa* indicated that about 70 per cent of the amino groups of the virus could be substituted without the loss of infectivity. Further reaction was accompanied by the inactivation of the virus. In general, 10 to 20 per cent of the phenol plus indole groups could be substituted without a decrease in activity. When tested on leaves of *Phaseolus vulgaris*, a number of the derivatives exhibited a significantly lower specific activity than when tested on leaves of *Nicotiana glutinosa*. When samples of each of the derivatives were inoculated into plants of Turkish tobacco and allowed to propagate, normal virus was formed. The results as a whole confirmed those previously obtained with the acetyl and phenylureido derivatives. It was therefore concluded that the nature of the substituent chemical radicals had rather little specific effect on the physiological behavior of the derivatives.

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## DERIVATIVES OF TOBACCO MOSAIC VIRUS

### III. THE RÔLE OF DENATURATION OF THE VIRUS IN THE MEASUREMENT OF PHENOLIC GROUPS

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In applying Herriott's methods (1) to the determination of phenolic groups in acetyl and phenylureido derivatives of tobacco mosaic virus, Miller and Stanley introduced urea into the mixtures of virus and color reagent in order to minimize turbidity (2). This was particularly important in the pH 11 method, since the virus became denatured at pH 11 and the urea was required to disperse the denatured protein. It was gradually realized, however, that in addition the urea exerted a denaturing influence on the virus under the conditions of the pH 8 method. This was indicated by the progressive disappearance of the opalescence characteristic of solutions of tobacco mosaic virus. The degree of denaturation of samples of the virus was found to determine the amount of color given by the virus in the pH 8 method. Furthermore, different preparations of virus were observed to vary in their rates of denaturation. Preparations of acetylated virus (2) were, as a rule, denatured at a rate comparable to that of normal virus. The phenylureido virus (2) and carbobenzoxy, *p*-chlorobenzoyl, and benzenesulfonyl derivatives more recently prepared (3) were, however, relatively more resistant to denaturation. It appeared necessary, therefore, to evolve a method by means of which a more uniform denaturation might be obtained.

The earlier work of Stanley and Lauffer on the denaturation of tobacco mosaic virus in urea (4) suggested that a more rapid and uniform denaturation should be brought about if the final pH of the mixtures of virus, urea, and color reagent was made slightly more alkaline. Preliminary studies showed this supposition to be correct. However, it was discovered during the course of these experiments that the pH of the mixtures gradually changed during the period of time allowed for the color development. For example, when the amounts of the phosphate buffer and Folin color reagent were adjusted to give a pH of 7.6 after 20 minutes of standing, the initial pH was 8.2. When the amounts were adjusted to give a pH of 7.8 after 20 minutes, the initial pH was as high as 9.4. In view of the lability of substituted phenolic linkages to alkali (5), the procedure under the less alkaline conditions represented by the first experiment appeared to be

preferable for measurements to be carried out on the derivatives of the virus. Unfortunately, however, a complete denaturation of the virus by means of the urea could not be expected at the lower pH.

*Development of Procedures with Sodium Dodecyl Sulfate As the Denaturing Agent in the Determination of Phenol Plus Indole<sup>1</sup> Groups*—The solution to the problem of obtaining complete denaturation of the virus without danger of hydrolysis of substituent groupings was found in the observation that the virus could be denatured rapidly and completely in weakly acid solution by means of the detergent, sodium dodecyl sulfate.<sup>2</sup> On this basis, a modified pH 8 method was worked out as follows: To 1 or 2 mg. of virus or virus derivative in 1 cc. of solution were added 0.2 cc. of 10 per cent sodium dodecyl sulfate and 0.1 cc. of 0.2 N hydrochloric acid. The opalescence of the mixture disappeared within a few seconds. The pH was 2.1. After 2 minutes, the mixture was neutralized with 0.1 cc. of 0.2 N sodium hydroxide, and 1 cc. of phenol reagent and 2 cc. of phosphate buffer were added. The buffer was made up of 9 parts of 0.5 M disodium phosphate and 1 part of 10 per cent sodium hydroxide. The phenol reagent before use was diluted so that 1 cc. of the reagent mixed with 2 cc. of the phosphate and 1 cc. of water would give a pH of 7.6 to 7.7 after 10 minutes. The final mixture of virus, detergent, and color reagents developed the characteristic blue color of the phenol test. Evidence that complete denaturation was obtained under these conditions was provided by the observation that, in the case of the normal virus, the same chromogenic power which was obtained above was also exhibited when the virus was heated with the acid detergent for 10 minutes at 100° or when it was first denatured with alkali under the conditions of the pH 11 method and then subsequently neutralized with acid and dispersed with detergent.

The stability to the acid detergent of the substituted phenolic linkages in the various derivatives of the virus was established by means of control experiments with the corresponding derivatives of tyrosine. For these tests, 1 cc. aliquots of solutions of diacetyl, diphenylcarbamido, dicarbo-benzoyl, dibenzoyl, and dibenzenesulfonyl derivatives of tyrosine, made up as described elsewhere (5), were each treated with detergent and

<sup>1</sup> It is a recognized fact that indole as well as phenol groups respond to the Folin color test. It appears, however, that in proteins the phenol groups are generally more affected by reagents such as ketene than are the indole groups. Since it is uncertain what proportion of the total chromogenic power of the unhydrolyzed virus towards the Folin reagent is contributed by the phenol groups and what proportion by the indole groups, it is necessary to measure the extent of substitution of phenol groups empirically in terms of phenol plus indole groups.

<sup>2</sup> We wish to thank Dr. S. Lenher of E. I. du Pont de Nemours and Company, Inc., for putting in our hands a generous supply of analytically pure sodium dodecyl sulfate.

hydrochloric acid as above, and were allowed to stand at room temperature for 60 minutes. The mixtures were then neutralized and the color reagents were added. The colors of the final solutions were compared with suitable standards. No significant hydrolysis of the substituted phenolic linkages was observed in any case except that of the acetyltyrosine, in which case only 3 per cent hydrolysis was observed. These results indicated that the hydrolytic cleavage of such linkages during the analysis of derivatives of the virus by the above procedures was improbable. It was therefore concluded that reliable results were obtainable by the modified pH 8 method described.

It was next of interest to determine whether the sodium dodecyl sulfate could be incorporated into the saponification procedure of the pH 11 method. It was first attempted to obtain saponification and denaturation of the virus preparations by treating 1 cc. of virus solution with 0.2 cc. of 10 per cent detergent and 0.1 cc. of 0.2 N sodium hydroxide. Under these conditions, the normal virus was quite readily denatured but preparations of certain of the derivatives, in particular those which previously had been found to be resistant to denaturation with urea, were very slowly denatured by the alkaline detergent. Furthermore, in control experiments with derivatives of tyrosine it was noted that the sodium dodecyl sulfate exerted an inhibitory effect on the saponification of the substituted phenolic linkages.

In view of the above results, it was concluded that the saponification procedure should be carried out in the absence of the detergent. The procedure was therefore as follows: To 1 cc. aliquots of virus solution were added 0.1 cc. portions of 0.2 N sodium hydroxide. The mixtures were allowed to stand at room temperature for the period of time which was found to be required for complete denaturation and saponification. A period of 2 minutes was usually sufficient for the normal virus; 20 minutes, for preparations of acetyl virus. Periods of 2 to 3 hours were allowed for samples of phenylureido, carbobenzoxy, *p*-chlorobenzoyl, or benzenesulfonyl derivatives. For reasons discussed in a separate communication (5), anomalous results were to be expected for the phenylureido and benzenesulfonyl derivatives, even though complete denaturation was obtained. 0.2 cc. of 10 per cent sodium dodecyl sulfate and 0.1 cc. of 0.2 N hydrochloric acid were then added to each sample. The mixtures were finally treated with the phenol reagent and phosphate buffer as described above for the pH 8 method. By this means satisfactory results were obtained in the pH 11 method.

It was considered important to establish whether the detergent might exert some specific untoward influence in the phenol color reaction. As a test for this possibility, the comparative effects of urea and sodium dodecyl



sulfate on the chromogenic power of normal virus and of tyrosine towards the phenol reagent were determined. For these measurements, the pH 11 procedure was employed essentially as described above. A complete denaturation of the virus was thus assured by the treatment with alkali. In order to obtain the same final volumes in each set of experiments, 2 cc. of 1 per cent detergent were used in the sodium dodecyl sulfate procedure and 2 cc. of 50 per cent urea were used in the urea procedure. After the reagent and phosphate were added, clear solutions were obtained in all tests except that of the virus in the presence of urea, in which case the slight turbidity which was present was removed by centrifugation. The colors were measured after 80 minutes. The virus gave 25 per cent more color in the urea procedure than in the procedure employing sodium dodecyl sulfate. The corresponding figure in the studies with tyrosine was 22 per cent. There is no reason to believe, however, that the results obtained in measurements carried out with the detergent as the dispersing agent are any less valid because of this difference.

*Application of Modified Procedures to Virus Derivatives and Comparison with Results Obtained by Original Methods*—In order to test the possibility that the apparent substitution of phenol groups by ketene and phenyl isocyanate reported earlier (2) may have represented artifacts which arose from an incomplete denaturation of the preparations of the derivatives under the conditions of the analytical methods employed at that time, determinations by the modified procedures indicated above were repeated on the original virus derivatives. A preparation of acetylated virus, which had appeared to have had 23 per cent of its phenol plus indole groups substituted (2), was found on check determination to be completely unsubstituted in this regard. Determinations of amino nitrogen carried out at the same time revealed, however, that the substitution of the amino groups was unchanged. The recent observation of Tracy and Ross (6) that malonyl substituent groups bound to the phenolic hydroxyl groups of horse serum albumin are very labile and subject to spontaneous hydrolysis led us to suspect that an analogous hydrolysis of acetyl-phenolic linkages had occurred in the acetylated virus during the period of approximately 7 months since the derivative was originally prepared. A fresh batch of acetylated virus was therefore prepared and was analyzed immediately for phenol plus indole groups by the modified analytical procedures. By the pH 8 method, the extent of substitution of these groups was found to be 28 per cent. By the pH 11 method, the acetylated virus yielded 95 per cent of the color given by the normal virus. The results of these experiments thus established the reliability of the corresponding results obtained in the earlier work and in addition demonstrated that the acetyl-phenolic

linkages in preparations of acetylated virus may undergo a gradual spontaneous hydrolysis.

A sample of phenylureido virus in which the phenol plus indole groups had previously appeared to have been substituted to the extent of 43 per cent (2) was found by the modified pH 8 procedure to be substituted only 17 per cent. When the analysis was repeated by the original procedure in which urea was employed, a high extent of substitution was again indicated, thus demonstrating that in the case of the phenylureido virus the lower result obtained by the modified procedure was due primarily to a more complete denaturation of the virus rather than to a spontaneous alteration in the virus which might have occurred during the period since it was first prepared.

As in the phenylureido virus, determinations of phenol plus indole groups in carbobenzoxy, *p*-chlorobenzoyl, and benzenesulfonyl derivatives of the virus indicated much higher apparent degrees of substitution by the urea procedure than by the modified procedure employing sodium dodecyl sulfate. For example, preparations in which the phenol plus indole groups appeared, by the urea method, to have been substituted to the extent of 30 to 40 per cent were found by the modified procedure to have been substituted only 10 to 20 per cent. These results serve as further evidence for the importance of securing complete denaturation.

A close analogy to the results which we have obtained with tobacco mosaic virus may be seen in studies of Herriott with pepsinogen (7). Herriott found that by the pH 8 method pepsinogen gave less color than did pepsin, whereas, by the pH 11 method, nearly the same color values were obtained with both proteins. These results were explained on the basis that only the pepsin was denatured at pH 8, whereas both pepsin and pepsinogen were denatured by the pH 11 treatment and therefore yielded their full complement of color under the conditions of the pH 11 method.

#### SUMMARY

The chromogenic power of preparations of tobacco mosaic virus towards the phenol reagent was found to depend upon the completeness with which the virus was denatured. Furthermore, different preparations of the virus or of its artificially prepared derivatives were observed to exhibit different rates of denaturation. By means of the use of sodium dodecyl sulfate as the denaturing agent, conditions were worked out which assured a uniform denaturation of the virus. The application of these findings to the measurement of phenol plus indole groups in derivatives of the virus was described and comparisons were made with the results obtained by the methods formerly employed.

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## DERIVATIVES OF TOBACCO MOSAIC VIRUS

### IV. A STUDY OF THE DETERMINATION OF PHENOL GROUPS IN VIRUS DERIVATIVES BY MEANS OF MODEL EXPERIMENTS WITH DERIVATIVES OF TYROSINE

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Measurements of phenol groups in acetyl derivatives of tobacco mosaic virus by Herriott's methods (1) have been found to be easily duplicated and apparently reliable (2, 3). The extension of the methods to certain other derivatives of the virus, however, led to unsatisfactory results, owing to the resistance of these derivatives to denaturation. It therefore became necessary to work out modified methods of analysis which would assure complete denaturation of each derivative (3). During the course of this work, it was observed that under the usual conditions of the pH 11 method (2) the carbobenzoxy, *p*-chlorobenzoyl, and benzenesulfonyl derivatives of the virus exhibited a much less complete recovery of chromogenic power than that shown by the acetyl virus. The phenylureido virus, on the other hand, exhibited in the pH 11 method even more chromogenic power than that of the normal virus. In an effort to explain these apparently anomalous results, studies on the behavior of the differently substituted phenolic linkages under the conditions of Herriott's methods were carried out by means of model experiments with the corresponding derivatives of free tyrosine.

The rates of saponification of disubstituted tyrosine derivatives at pH 11 were first determined. Complete hydrolysis of a given derivative was assumed to have been obtained when the chromogenic power reached a maximum and remained constant. The results are presented graphically in Fig. 1 from which it may be seen that the diphenylcarbamidotyrosine, a derivative which corresponded to the phenylureido virus, was completely saponified within 30 seconds and the diacetyltyrosine, within about 10 minutes. The dicarbenzoxy and dibenzoyl derivatives, on the other hand, required nearly 30 minutes. In still greater contrast, the dibenzenesulfonyl derivative was not measurably saponified even after 60 minutes. When heated at 100° at pH 11, the dibenzenesulfonyltyrosine required over 30 minutes for saponification, whereas the other derivatives of tyrosine were saponified within 1 minute. Abderhalden and Bahn reported that the benzenesulfonyl-phenolic linkage of dibenzenesulfonyltyrosine is very stable to alkali (4). It was apparent from the above results that the low

chromogenic power exhibited by the carbobenzoxy, *p*-chlorobenzoyl, and benzenesulfonyl derivatives of tobacco mosaic virus when analyzed by the pH 11 method could have been due, in part at least, to the slow rate of saponification of the substituted phenolic linkages involved.

More detailed studies were next carried out with phenylcarbamido derivatives of tyrosine. The diphenylcarbamidotyrosine, after treatment at pH 11, was found to yield with the phenol reagent an amount of color which was about 16 per cent *greater* than that yielded by an equimolecular amount of tyrosine. The monophenylcarbamidotyrosine, on the other hand, yielded approximately 22 per cent *less* color than an equivalent amount of tyrosine. Since it was to be expected that aniline would be formed as a product of the saponification of the diphenylcarbamidotyrosine, it appeared that this substance was responsible for the extra chromogenic power possessed by the disubstituted tyrosine. In colorimetric tests car-

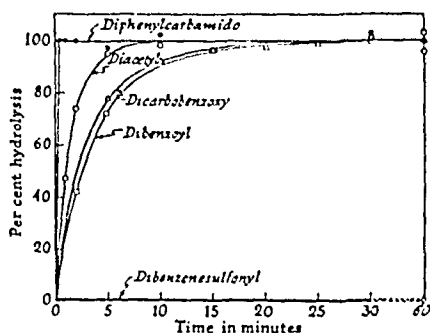


FIG. 1. Rates of saponification of derivatives of tyrosine

ried out on aqueous solutions of doubly distilled aniline, the compound was found to give with the phenol reagent about 35 per cent of the color given by an equimolecular amount of tyrosine. It was apparent that this amount of color accounted for the difference in the chromogenic power of the monophenylcarbamidotyrosine and that of the saponified diphenylcarbamido derivative. It was therefore concluded that, by analogy, the extra color yielded by phenylureido derivatives of tobacco mosaic virus after treatment at pH 11 was also due to the presence of aniline which was formed during the saponification of the derivatives.

The direct application of the results of the model experiments to the determination of phenol groups in derivatives of tobacco mosaic virus did not give rise to completely satisfactory results in all cases. This was due to the secondary effect of the slow rate of denaturation of certain preparations of derivatives of the virus under the conditions of the pH 11 method (3). In the cases of the acetyl, carbobenzoxy, and *p*-chlorobenzoyl deriva-

tives of the virus, it was found, however, that a complete denaturation and saponification could be obtained if the pH 11 treatment was carried out at 100° for a period of 2 minutes. This procedure appeared to be justified on the basis of the finding that under these more vigorous conditions samples of normal virus exhibited the same chromogenic power which they exhibited when denatured by acid or alkaline detergent at room temperature. Determinations of the phenylureido virus or benzenesulfonyl virus by the pH 11 method were carried out by saponification at room temperature for 2 or 3 hours (3). Because of the properties of the linkages involved, the appearance of an excess of chromogenic power in the phenylureido virus and a complete lack of recovery of chromogenic power in the benzenesulfonyl virus served as corroborative evidence for the substitution of phenolic linkages within these particular derivatives.

TABLE I  
*Relative Chromogenic Powers of Different Derivatives of Tyrosine*

Compound	Relative chromogenic power (pH 11 method)
Tyrosine.....	100
N-Glycyltyrosine.....	89
N-Phenylcarbamidotyrosine.....	78
O,N-Diacetyltyrosine.....	78
O,N-Dibenzoyltyrosine.....	80
O,N-Dicarbobenzoxytyrosine.....	75
N-Carbobenzoxytyrosine.....	78*
N-Chloroacetyltyrosine.....	83*
N-Carbobenzoxytyrosylglycine.....	87*

\* Data of Tracy and Ross (5).

It was observed in the course of the experiments with the derivatives of tyrosine that the chromogenic values given by the various derivatives in the pH 11 method did not coincide with that given by free tyrosine. Because of the stability of the linkage between the various substituent radicals and the amino group of the tyrosine, the chromogenic power of the saponified disubstituted derivatives was due in general to the monosubstituted derivatives which were formed. In Table I, the relative chromogenic powers of the different tyrosine derivatives, together with that of a sample of glycyltyrosine, are compared with the chromogenic power of free tyrosine. Similar data obtained by Tracy and Ross (5) for a number of other derivatives of tyrosine also are included in Table I. Without exception, the derivatives developed less color with the Folin reagent than did equivalent amounts of tyrosine. Tracy and Ross concluded that, in general, substitution diminishes the color developed by

tyrosine with the phenol reagent. Our present data serve as additional evidence for this generalization. It was of particular significance in this connection that tyrosine appears to possess a diminished chromogenic power when linked within the intact protein molecule. For example, it was found by Herriott that intact proteins when treated with the Folin reagent yielded on the average only 59 per cent of the color to be expected from their known content of tyrosine and tryptophane (1). Other investigators have obtained similar results (2, 5-7). In view of the data obtained with tyrosine derivatives of known structure, it might well be anticipated that the units of tyrosine which occur within protein molecules would exhibit a diminished reaction with the Folin reagent.

#### EXPERIMENTAL

*Preparation of O,N-Diphenylcarbamidotyrosine*—5 gm. of tyrosine dissolved in a mixture of 100 cc. of 2 per cent sodium hydroxide and 100 cc. of 1 M dipotassium phosphate were treated with continuous stirring at 0° with 8 cc. of phenyl isocyanate added in 2 cc. portions at 10 minute intervals. 12 cc. of 10 per cent sodium hydroxide were added gradually during the reaction to maintain the pH at 8 to 9. Diphenylurea was extracted with ether and the aqueous layer was acidified with hydrochloric acid. The precipitate which formed was filtered and dried. The yield, 15.8 gm., was practically quantitative. When crystallized from hot 95 per cent ethyl alcohol, the compound separated as spear-shaped plates, which, after a second recrystallization, melted at 205-206° with effervescence. Neutralization equivalent, 421; calculated for diphenylcarbamidotyrosine, 419. C 65.6, H 5.0, N 10.0 per cent; theory, C 65.9, H 5.0, N 10.0 per cent. The high sensitivity of the phenylcarbamido-phenolic linkage to alkali probably accounted for the failure of Gaunt and Wormald to obtain this compound under the conditions they employed (8).

*Preparation of N-Phenylcarbamidotyrosine*—5 gm. of tyrosine dissolved in 200 cc. of 1 per cent sodium hydroxide were treated with continuous stirring at 0° with 4 cc. of phenyl isocyanate added in 0.5 cc. portions at 5 minute intervals. 4 cc. of 10 per cent sodium hydroxide were added during the reaction to maintain the solution strongly alkaline to phenolphthalein. The mixture was washed with ether, filtered, and acidified with hydrochloric acid. The product separated as an oil which crystallized as long, flat needles on cooling and stirring. The yield was 7.4 gm. (89 per cent of the theory). After repeated recrystallization from dilute alcohol, the compound softened at 104° and melted with effervescence at 106-110°. Beilstein (9) has given a melting point of 104° for N-phenylcarbamidotyrosine. Neutralization equivalent, 298; calculated for N-phenylcarbamidotyrosine, 300.

*Preparation of Diacetyl, Dicarbobenzoxy, Dibenzoyl, and Dibenzenesulfonyl*

*Derivatives of Tyrosine*—A sample of diacetyltyrosine, prepared by treating tyrosine with ketene (10), was obtained through the courtesy of Dr. R. M. Herriott. The dicarbobenzoxo, dibenzoyl, and dibenzenesulfonyl derivatives of tyrosine were prepared by procedures described elsewhere (4, 11–14).

*Measurements of Rates of Saponification of Tyrosine Derivatives*—For solutions of the diacetyl, dibenzoyl, and dibenzenesulfonyl derivatives, weighed samples were dissolved in small volumes of acetone, neutralized with the calculated amounts of 0.05 N sodium hydroxide, and diluted with water to the desired final volumes. For the solution of the diphenylcarbamido derivative, the weighed sample was suspended in a little dilute acetone and was dissolved by the addition of the minimum required amount of 0.05 M disodium phosphate. For the preparation of the solution of dicarbobenzoxo-tyrosine, a much larger amount of acetone was required in order to keep the neutralized derivative in solution. The acetone did not interfere with the color tests. Each of the solutions was made up to contain an amount of derivative approximately equivalent to 0.1 mg. of tyrosine per cc.

To 1 cc. aliquots of each solution was added 0.1 cc. of 0.2 N sodium hydroxide. The mixtures were allowed to stand at 25° or at 100° for the desired period of time and were then neutralized with 0.1 cc. of 0.2 N hydrochloric acid. 0.2 cc. of 10 per cent sodium dodecyl sulfate and 0.6 cc. of water were added, followed by 1 cc. of Folin reagent and 2 cc. of alkaline phosphate buffer. The alkaline buffer and Folin reagent were prepared as described in a previous paper (3). The colors were measured in the Klett-Summerson colorimeter at the end of 15 minutes.

The use of sodium dodecyl sulfate (3) in the place of urea formerly employed (2) has no special significance as far as the above experiments are concerned. It was of interest to observe, however, that the detergent possessed a remarkable dispersing power for the tyrosine derivatives which were relatively insoluble in water.

#### SUMMARY

Carbobenzoxo, *p*-chlorobenzoyl, and benzenesulfonyl derivatives of tobacco mosaic virus were found to give a less complete recovery of chromogenic power under the usual conditions of the pH 11 method of Herriott than did the acetyl derivative of the virus. By means of model experiments with the corresponding derivatives of tyrosine, it was demonstrated that the different substituent radicals on the phenolic group varied considerably in their rates of saponification. The application of the findings to the measurement of phenolic groups in derivatives of tobacco mosaic virus was discussed.

The phenylureido derivative of tobacco mosaic virus was found to yield



more color in the pH 11 method than did normal virus. By means of studies on the chromogenic power of pure aniline and on the behavior of N-phenylcarbamido and O,N-diphenylcarbamido derivatives of tyrosine, this result was demonstrated to be due to the formation of aniline during the treatment at pH 11.

Monosubstituted tyrosine derivatives were found to yield with the phenol color reagent less color than did free tyrosine. An analogy was pointed out between this property of known derivatives of tyrosine and the similar low chromogenic power of tyrosine when present in protein linkage.

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# INACTIVATION OF BIOTIN BY RANCID FATS\*

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In studies involving the feeding of diets containing cod liver oil and butter fat to rats, some of the animals developed typical symptoms of mild biotin deficiency, *i.e.* spectacle eye and spasticity of gait, after being maintained on the diet 12 to 16 weeks. It was soon evident that such a ration containing cod liver oil and butter fat was very prone to turn rancid and that this rancidity was responsible for rapid losses of vitamin A. This loss of vitamin A has been previously noted by other workers (1, 2) but the practice of incorporating cod liver oil in diets is still prevalent in many laboratories. Destruction of other vitamins in rations containing fish liver oils has been demonstrated by Fritz *et al.* (3) in the case of vitamin D and by Macomber (4) and Cummings and Mattill (5) for vitamin E. In this paper the destruction *in vitro* of one of the B vitamins, biotin, by rancid diets and fats of a high peroxide number will be reported.

## EXPERIMENTAL

The general procedure employed in studying the inactivation of biotin by various agents was as follows: The material to be studied was emulsified in 2 per cent gum ghatti solution, a few drops of toluene and the biotin solution added (total volume, 10 cc.), and the mixture placed in a glass-stoppered, 50 cc., round bottom flask in a mechanical shaker while being incubated. Incubation of a few preliminary runs was made at room temperature but, since incubation at 37° gave more rapid inactivation and in addition represented more nearly the intestinal temperature of the rat, most of the experiments were conducted at the higher temperature. After incubation the entire contents of the flask were diluted to a suitable volume and the amount of biotin determined by the microbiological method with *Lactobacillus casei* of Shull, Hutchings, and Peterson (6).

The biotin employed in most of the experiments was a biotin concentrate (S. M. A. No. 5000), although several experiments were made with the pure crystalline biotin free acid (S. M. A.).

*Experiments with Rancid Rations*—The ration consisting of 73 per cent sucrose, 18 per cent Labco casein, 4 per cent Salts 4 (7), 3 per cent filtered

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

butter fat, and 2 per cent cod liver oil was allowed to turn rancid by being kept standing at room temperature for 1 week. The degree of rancidity was followed by determination of the peroxide number of the fat extracted from the diet with purified ethyl ether. The fat from freshly made ration possessed a peroxide number of 7 (which was due to the slight rancidity present in the cod liver oil), while fat from the rancidified diet gave a value of 49. If 10 gm. of the rancid ration were incubated with the biotin, definite destruction of this factor could be noted. Incubation at room temperature for 24 hours resulted in 20 per cent of the biotin being destroyed (inactivated). That the active principle responsible for the inactivation resided in the fat was demonstrated by noting no inactivation when the ether-extracted ration was used. The ether extract of the ration was then concentrated and the fat emulsified and incubated with biotin. In this case, biotin inactivation equal to that shown by the original rancid ration was observed. As mentioned previously the peroxide number of the rancid fat was 49 and, since it is impractical to increase this value much further, aerated corn oil (Mazola) of a peroxide number of 200 was used to study the effects of increased rancidity on the inactivation of biotin. Crystalline biotin in lower concentration was used in this latter experiment; so the results cannot be compared directly with the previous trials in which higher biotin concentrations were used, but in general it has been observed that the higher the peroxide number of the fat, the more rapid is the destruction of biotin under these experimental conditions. Table I, Experiments 1 to 8, summarizes the above results.

*Experiments with Aerated Ethyl Linolate*—To obtain a fat of a higher degree of purity than the butter fat-cod liver oil combination or the aerated corn oil, distilled samples of ethyl linolate<sup>1</sup> were aerated at 37° for 48 hours or until the peroxide number reached 500 or higher. Experiments 9 to 11, Table I, give the results obtained when aerated ethyl linolate of a peroxide number of 575 was used as the biotin-inactivating agent. It can be seen that the inactivation was more nearly complete and occurred more rapidly than was noted with the fats of a lower peroxide number.

*Effect of  $\alpha$ -Tocopherol on Biotin Inactivation*—The stabilizing effects of antioxidants, especially of vitamin E, on carotene have been reported by Quackenbush, Cox, and Steenbock (8). With the ration described above it has been shown<sup>2</sup> that small amounts of  $\alpha$ -tocopherol stabilized vitamin A. In view of these experiments it was of interest to see whether this compound exerted any similar stabilization of biotin in the presence of rancid fat.

In this series of experiments pure biotin (free acid) was used. The  $\alpha$ -tocopherol (20 mg. per 500 mg. of fat) was added to the rancidified ethyl

<sup>1</sup> Prepared from corn oil by Mr. Raymond Thompson.

<sup>2</sup> Pavcek, P. L., unpublished data.

linolate and allowed to stand at room temperature for 8 hours before the addition of the biotin and incubation. (The ethyl linolate without vitamin E added was likewise allowed to stand 8 hours before addition of the biotin.) It had been noted by preliminary trial that if the tocopherol were added directly to the ethyl linolate-biotin emulsion it had very little effect on biotin stabilization, owing probably to poor contact with the ethyl linolate. Samples were withdrawn at 0, 12, 24, and 48 hour intervals and the amount of biotin remaining was determined. The results are presented in Fig. 1. It can be seen that the destruction of biotin is greatly reduced by the presence of the  $\alpha$ -tocopherol, 60 per cent of the amount originally

TABLE I  
*Inactivation of Biotin by Various Agents*

Experiment No. and inactivating agent	Peroxide No. of agent	Incubation		Biotin added	Biotin found	Per cent inacti- vation
		Time	Tem- perature			
		hrs.	°C.	micro- micrograms	micro- micrograms	
1. 10 gm. rancid ration		24	25	3958	3170	20
2. 10 " ether-extracted ran- cid ration		24	25	3958	3958	0
3. 500 mg. rancid fat from (2)	49	24	25	3120	2780	11
4. 500 " " "	49	24	37	3355	2425	27.5
5. 500 " " "	49	72	37	3355	2025	39.5
6. 500 " " "	49	168	37	3355	712	79
7. 500 " mineral oil		168	37	3355	3220	4
8. 500 " rancid corn oil	200	48	37	400*	43	89
9. 0.1 cc. ethyl linolate	575	24	37	3355	2650	20
10. 0.5 " " "	575	24	37	3355	632	81
11. 0.5 " " "	575	48	37	3355	39	99

\* Pure crystalline free acid.

present remaining after a 48 hour incubation. Without the added tocopherol 96 per cent of the biotin was inactivated within the first 12 hours.

*Nature of Inactivated Product*—Since fats of a high peroxide number seem to be responsible for the inactivation of biotin, the reaction is probably one of oxidation. Nielsen *et al.* (9) have shown that hydrogen peroxide-treated biotin although demonstrating no activity when measured by the *Lactobacillus casei* method still possessed 50 to 90 per cent potency when assayed by the yeast growth method (10). Thus it is important to know whether biotin which had been inactivated by the action of rancid fat also gave a response when measured by this latter method. Samples of the series without tocopherol (Fig. 1) were assayed by this means.<sup>3</sup> Instead of 99 per

<sup>3</sup> The assays were performed by Miss Josephine Gardner.

cent destruction of the biotin after 24 and 48 hours only 44 per cent destruction was indicated when these same samples were assayed by the yeast growth method. The interpretation that can be placed on these results is that the mechanism of biotin inactivation in the case of rancid fats is probably similar to that reported for weak solutions of hydrogen peroxide; *i.e.*, a partial oxidation of the sulfur moiety of the biotin molecule to a sulfoxide which can probably be reduced to biotin free acid by yeast but not by *Lactobacillus casei*.

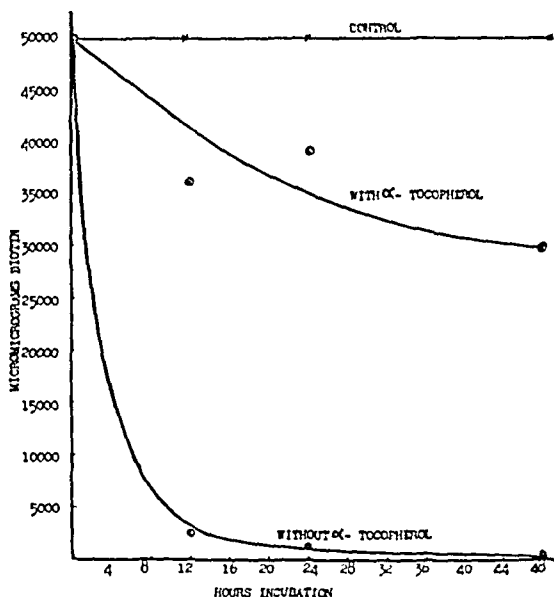


FIG. 1. Inactivation of biotin by rancidified ethyl linolate

#### DISCUSSION

From the above experiments it would appear that biotin in the form of concentrates or the pure free acid can be inactivated by rancidified fats. The mechanism of this reaction seems to be one of partial oxidation of biotin, in which form biotin is not detected by the *Lactobacillus casei* assay but does give a response by the yeast growth method. Although the inactivated product has not been isolated, it is highly probable that treatment of biotin with rancid fats or with dilute (0.3 per cent) hydrogen peroxide results in the same end-product.

No quantitative relationship with the high peroxide number of the fat has been attempted but in general the results indicate that the higher the peroxide number the greater is the inactivation observed. Such factors

as the presence of antioxidants will, no doubt, as in the case of carotene inactivation (8), alter the picture considerably.

The relationship of these findings to the production of biotin deficiency in animals is now being investigated and will be reported later.

#### SUMMARY

The inactivation *in vitro* of biotin by a rancidified ration and by fats and ethyl linolate of a high peroxide number has been studied. 96 per cent inactivation of pure biotin could be accomplished in 12 hours by means of ethyl linolate of a high peroxide number. In the presence of  $\alpha$ -tocopherol this inactivation amounted to only 40 per cent after 48 hours incubation. The inactivated product still showed 56 per cent activity when measured by the yeast growth method, indicating a similarity between the product formed from biotin by the action of rancid fat and by dilute hydrogen peroxide.

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# THE INHIBITION OF RAT GROWTH BY NICOTINAMIDE

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When fed to rats, dogs, or man, nicotinic acid and nicotinamide are excreted largely as trigonelline (1-3). No information has been available concerning the source of the necessary methyl group or the site of the methylation. Several attempts in these laboratories to demonstrate trigonelline synthesis *in vitro* with surviving liver, kidney, and muscle slices have failed to yield convincing, positive results. In the present study it has been found that the ingestion of relatively large amounts of nicotinamide inhibits the growth of young rats and that this effect can be prevented by the administration of methionine.

## EXPERIMENTAL

*Addition of Nicotinamide to an Arachin Basal Diet*—The basal diet for this experiment consisted of arachin 15, rice starch 30, sucrose 35, cottonseed oil 15, and salts (4) 5 parts. All rats received a daily supplement of 20  $\gamma$  of thiamine chloride, 20  $\gamma$  of pyridoxine, 40  $\gamma$  of riboflavin, 240  $\gamma$  of calcium pantothenate, 10  $\gamma$  of 3-methyl-1,4-naphthoquinone diacetate, and 2 drops daily of an equal mixture of wheat germ and cod liver oils. The arachin was prepared from peanut meal by the procedure of Johns and Jones (5). Four groups of six male rats each, all weighing between 48 and 54 gm. were employed. Group A received the basal diet alone; Group B received, in addition, 0.3 per cent of *dl*-methionine; Groups C and D were given 0.3 per cent of *dl*-methionine<sup>1</sup> plus 1.0 per cent of nicotinamide.<sup>1</sup> The rats were allowed to eat *ad libitum*. After 24 days another 0.6 per cent of *dl*-methionine was added to the ration of Group C, while 0.3 per cent of choline chloride<sup>1</sup> and 0.1 per cent of NaHCO<sub>3</sub> were added to the diet of Group D.

The results of this experiment are shown in Fig. 1. The basal diet supported very slow growth, 0.25 gm. per rat per day, while inclusion of methionine in the diet of Group B increased this rate to 1.0 gm. per rat per day. The presence of nicotinamide in the diet of Group C limited growth to but 0.4 gm. per rat per day. When more methionine was added to the diet of Group C, growth proceeded at the rate of 1.5 gm. per rat per day for the next 16 day period. The presence of choline in the diet of Group

<sup>1</sup> Merck and Company, Inc., Rahway, New Jersey.



D appeared to have only a very slight growth-stimulating action. When the rats were sacrificed, only the animals on the basal ration (Group A) appeared to have grossly fatty livers.

*Addition of Nicotinamide to Low Casein Diet*—The basal ration used in the next group of experiments contained casein 10, cottonseed oil 10, su-

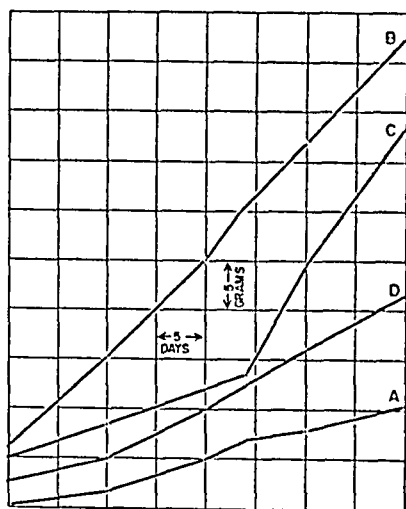


FIG. 1

Fig. 1. The effect of nicotinamide on the growth of rats receiving an arachin basal diet. Each curve is a composite of the growth of six rats. The supplements to the basal ration were as follows: Curve A none, Curve B 0.3 per cent of methionine, Curves C and D 0.3 per cent of methionine and 1.0 per cent of nicotinamide. At the arrows another 0.6 per cent of methionine was added to the ration of Group C (Curve C) and 0.3 per cent of choline to that of Group D (Curve D).

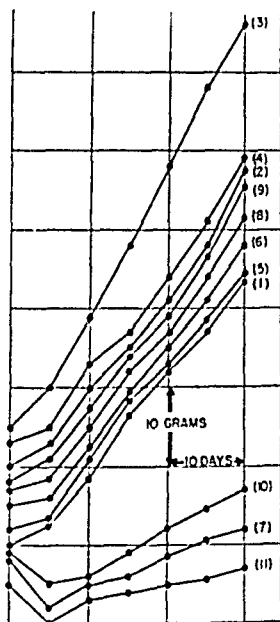


FIG. 2

Fig. 2. The effect of nicotinamide on the growth of rats. Each curve is a composite of the growth of twelve or eighteen rats. The supplements to the 10 per cent casein basal diet were as follows: Curve 1 none, Curve 2 choline, Curve 3 methionine, Curve 4 homocystine, Curve 5 nicotinic acid, Curve 6 nicotinic acid and choline, Curve 7 nicotinamide, Curve 8 nicotinamide, choline, and homocystine, Curve 9 nicotinamide and methionine, Curve 10 nicotinamide and choline, Curve 11 nicotinamide and homocystine.

crose 73, and salts (4) 7 parts. The vitamin supplement was identical with that described above. All rats were of the Vanderbilt strain (6) and weighed between 48 and 52 gm. at the start of the experiment. Because of the large number of groups involved, it was impossible to use one member of each litter in each group in the usual fashion, but in no case were two

rats from the same litter included in the same dietary group. Each group consisted of six rats and the entire experiment was performed three times. The growth curves shown in Fig. 2 are all composites of the total number of rats used on each experimental diet as are also the mean figures reported in Table I. Every addition to the basal ration was accompanied by a

TABLE I  
Effect of Nicotinamide on Rat Growth

Dietary supplement	Amount	Final weight	Liver weight	Liver fatty acids	Food intake	Weight gains*
	<i>per cent</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	<i>gm. per day</i>	<i>gm. per day</i>
None.....		83	6.0	17.2	6.6	1.10
<i>dl</i> -Methionine.....	0.6	99	4.5	4.5	7.6	1.63
Choline chloride.....	0.15	86	3.8	4.2	7.1	1.20
<i>dl</i> -Homocystine†.....	0.6	85	7.2	25.0	7.4	1.17
Nicotinic acid.....	1.0	81	5.8	24.1	7.3	1.03
"    ".....	1.0	83	4.5	4.9	7.2	1.10
Choline chloride.....	0.15					
Nicotinic acid.....	1.0	83	4.1	4.1	7.1	1.10
Betaine chloride.....	0.15					
Nicotinamide.....	1.0	54	4.8	8.5	4.2	0.13 (0.40)
"    ".....	1.0	86	4.0	5.0	6.7	1.20
<i>dl</i> -Methionine.....	0.6					
Nicotinamide.....	1.0	58	2.9	5.1	4.3	0.25 (0.48)
Choline chloride.....	0.3					
Nicotinamide.....	1.0	51	4.5	13.4	4.0	0.07 (0.24)
<i>dl</i> -Homocystine†.....	0.6					
Nicotinamide.....	1.0	84	3.9	4.5	6.5	1.13
Choline chloride.....	0.15					
<i>dl</i> -Homocystine.....	0.6	58	3.3	5.2	4.1	0.18 (0.32)
Nicotinamide.....	1.0					
Betaine chloride.....	0.3	52	4.9	14.1	4.3	0.0 (0.19)
Nicotinamide.....	1.0					
<i>l</i> -Cystine.....	0.6	82	7.1	24.2	4.3	1.07
Trigonelline sulfate.....	1.0					

\* Figures in parentheses represent the growth rate after the initial weight loss, rather than during the entire experimental period.

† S. M. A. Corporation, Chagrin Falls, Ohio.

corresponding decrease in the sucrose component. The additions to the basal diet and other pertinent data are summarized in Table I. The composite growth curves for the more important groups of rats are shown in Fig. 2. Each experiment has been duplicated with the same number of female rats, but for economy of space the results are not included since qualitatively the two series were, in all respects, identical.

While nicotinic acid had no effect on the growth of these rats, nicotinamide fed at the same level strongly inhibited growth. However, the nicotinic acid did seem to increase the fat content of the livers even beyond the already high level produced by the low casein basal diet. The addition of either choline or betaine to a nicotinic acid-containing diet completely prevented the formation of fatty livers and had a faint accelerating action on growth. The livers of the rats that had been given nicotinamide contained about half as much fat as that seen in the livers of the basal controls. The rats fed methionine with the nicotinamide grew at a rate slightly greater than that of the basal control rats and their livers contained normal amounts of fat. Both cystine and homocystine were unable to restore growth in the presence of nicotinamide and appeared to result in fattier livers than did the nicotinamide alone. While choline and betaine had little, if any, growth-stimulating action in the presence of nicotinamide, both of these substances completely prevented the formation of fatty livers. On the other hand, when choline and homocystine were fed together with nicotinamide, growth proceeded at the normal rate and no fatty liver formation was observed in these rats. The ingestion of trigonelline had no effect on growth although it did seem to increase the liver fat content of the male rats.

Since methionine was found to counteract the growth inhibition which resulted from the feeding of nicotinamide and since ingested nicotinamide is known to be excreted in part as the methyl betaine, trigonelline, it was thought worth while to investigate the trigonelline excretion of rats under several of the dietary conditions already described. For this purpose three rats from each group were placed in metabolism cages after 14 days on their experimental diets and urine was collected under toluene for a 3 day period. The pooled urine samples were then analyzed for total nicotinic acid and trigonelline (7, 8). For these analyses we are indebted to Mr. Jesse Huff, Dr. H. P. Sarett, and Dr. W. A. Perlzweig. The results are summarized in Table II.

The very poor urinary recovery of the ingested nicotinic acid and nicotinamide would appear to invalidate any positive conclusions from these data. These low recoveries are in agreement with the findings of Huff and Perlzweig who have found that as much as 40 per cent of administered trigonelline cannot be recovered in the urine of rats (1). No evidence has been advanced to elucidate the fate of the unrecovered nicotinic acid or nicotinamide. Nevertheless, from the data in Table II it is obvious that the rats did synthesize much more trigonelline when given more dietary methionine. Further, it does seem that the rat can synthesize trigonelline more readily from nicotinamide than from the free acid, as indicated by comparison of the trigonelline to nicotinic acid ratio in each case. More-

over, while the rats receiving nicotinamide ate less than half as much as the rats receiving nicotinic acid, these groups excreted equal quantities of trigonelline. These differences may well account for the difference in the behavior of nicotinic acid and nicotinamide in their effects on growth.

That nicotinic acid does not inhibit the growth of young rats has been reported by Unna (9). Since the difference between the acid and the amide may be quantitative rather than qualitative, several feeding trials were conducted with 2 per cent of nicotinic acid in a 10 per cent casein diet. At this concentration, nicotinic acid appeared to have a small but definite inhibitory action.

To test the possibility of strain differences, the growth of two strains of Wistar rats fed both nicotinic acid and nicotinamide as 1 per cent sup-

TABLE II

*Excretion of Trigonelline and Acid-Hydrolyzable Nicotinic Acid Derivatives by Rats on Diets Containing Large Quantities of Nicotinic Acid or Nicotinamide*

All values are expressed in terms of one rat for 1 day.

Dietary supplement	Food intake	Methionine in food	Nicotinic acid intake	Nicotinamide intake	Urine nicotinic acid	Urine trigonelline	Trigonelline	Recovery of ingested nicotinic acid or nicotinamide
	gm.	mg.	mg.	mg.	mg.	mg.	per cent of total	per cent
None.....	6.2	15	0	0	0.024	0.095	80.0	
Nicotinic acid.....	8.7	21	87	0	37.0	3.1	7.7	46.1
"    + choline chloride.....	7.9	19	79	0	23.2	2.4	9.3	32.4
Nicotinamide.....	3.2	8	0	32	8.5	2.3	21.3	33.8
"    + methionine.....	7.4	62	0	74	10.8	7.1	40.0	24.2
"    + choline chloride.....	5.0	12	0	50	9.2	3.2	25.8	22.8

plements to a 10 per cent casein diet was studied. Both strains behaved in the manner described for the Vanderbilt rats.

The basal diet used in the preceding experiments always resulted in the production of fatty livers unless supplemented with choline or methionine. To observe better the effect of nicotinic acid and nicotinamide in producing fatty livers, several groups of six male rats each were put on a 20 per cent casein diet for 14 days. Pooled urine samples were collected under toluene during the last 2 days of the experimental period. The basal diet consisted of casein 20, cottonseed oil 10, rice starch 30, sucrose 33, and salts (4) 7 parts. The vitamin supplement was identical with that previously described. The results are summarized in Tables III and IV.

Nicotinic acid exerted no inhibitory effect on growth under these con-

ditions, but the livers of the rats in this group were decidedly fatty, although the diet contained only a moderate amount of fat. The ingestion of nicotinamide induced a sharp weight loss but had no effect on the fat content of the livers of these animals. This is in agreement with the fact, observed by us under other conditions, that fatty livers cannot be induced

TABLE III

*Effect of Addition of Nicotinamide and Nicotinic Acid to a 20 Per Cent Casein Diet*

Dietary supplement	Initial weight	Weight change	Food intake	Liver	Liver fatty acids
	gm.	gm. per day	gm. per day	gm.	per cent
None.....	127	3.4	13.2	8.53	4.2
2% nicotinic acid.....	119	3.4	13.1	7.90	11.8
2% nicotinamide.....	120	-1.8	7.6	4.73	4.8
2% nicotinic acid + 0.4% choline chloride.....	121	3.0	12.3	7.37	4.1
2% nicotinamide + 0.4% choline chloride.....	119	-1.7	6.4	4.53	3.7
0.5% trigonelline (male).....	112	4.2	14.4	7.47	8.5
0.5% " (female).....	132	2.0	12.0	6.25	4.0

TABLE IV

*Urinary Excretion of Nicotinic Acid and Trigonelline*

All values are expressed in terms of one rat for 1 day.

Dietary supplement	Food intake	Nicotinic acid	Trigonelline	Trigonelline	Recovery
	gm. per day	mg.	mg.	per cent of total	per cent
None.....	13.7	0.025	0.090	78	
2% nicotinic acid.....	15.5	244	8.7	3.4	81
2% nicotinamide.....	8.3	108	7.8	6.7	70
2% nicotinic acid + 0.4% choline chloride.....	14.0	183	7.7	4.0	68
2% nicotinamide + 0.4% choline chloride.....	7.5	102	11.0	9.7	75
0.5% trigonelline (male).....	16.0	1.8	48.3	96.4	56
0.5% " (female).....	12.1	1.8	57.2	97.0	86

by dietary means in animals that are losing weight, are in negative nitrogen balance, and are ingesting insufficient food, especially if the diet contains no more than 10 per cent of fat.

Although choline had no effect on the growth of rats ingesting nicotinamide, it completely prevented the formation of fatty livers in the presence of nicotinic acid.

In agreement with the findings in Table I trigonelline appeared to increase the liver fat of male rats but had no effect upon the female rats. Although no explanation is available to account for this effect of trigonelline upon liver fat, the sex difference parallels the findings that male rats are more susceptible to choline deficiency (10) and that nicotinic acid can inhibit the growth of certain male rats but not their female litter mates (11). A large amount of the fed trigonelline could not be recovered in the urine. The appearance in the urine of considerable quantities of acid-hydrolyzable nicotinic acid derivatives after administration of trigonelline indicates that rats have some capacity for demethylating trigonelline. However, the appearance of fatty livers in these experiments and the inability of trigonelline to methylate homocystine (12) indicate that the methyl group of trigonelline is not available to the rat for transmethylation reactions.

TABLE V  
*Effect of Various Levels of Nicotinamide Ingestion on Rat Growth*

Nicotinamide supplement	Initial weight	Final weight	Change in weight	Food intake	Liver fatty acids
<i>per cent</i>	<i>gm.</i>	<i>gm.</i>	<i>gm. per day</i>	<i>gm. per day</i>	<i>per cent</i>
0	114	198	3.25	14.2	4.3
0.1	113	190	3.05	13.7	4.5
0.25	115	186	2.75	12.7	8.7
0.5	112	169	2.15	12.1	15.8
1.0	119	153	1.30	9.9	9.5
2.0	120	102	-0.7	7.6	5.0

Table V summarizes the effects of feeding nicotinamide at various levels as a supplement to the 20 per cent casein diet described above. This basal diet contained no choline. Six male rats were used in each group and the experiment was continued for 28 days. With increasing levels of nicotinamide the growth rate progressively decreased. At the 0.5 per cent level rat growth was seriously retarded and the livers were extremely fatty. At the 1.0 per cent level, nicotinamide strongly inhibited growth and the livers were but moderately fatty. The presence of 2.0 per cent nicotinamide produced an actual loss in weight over the entire experimental period and the livers of these rats appeared to be quite normal with respect to their fat content, albeit not quite large enough for rats of their age and weight in this colony.

This experiment again demonstrates that fatty livers can only be obtained in choline deficiency when all other dietary factors are close to optimal for normal growth. This finding suggests that the presence of fatty

livers in rats fed insufficient choline but adequate thiamine (13), riboflavin (14), and pantothenic acid (15) should not necessarily be regarded as positive effects due to the presence of these specific factors. Rather, the failure of fatty livers to appear in the absence of these essential substances may be only particular instances of the same growth failure phenomenon.

It seemed possible that while fatty livers were not apparent after 14 days of losing weight on the 2 per cent nicotinamide diet, they might have been present during the first few days on the diet and that the fat later slowly disappeared. To test this possibility another group of twenty-four male rats was fed the 2 per cent nicotinamide, 20 per cent casein diet *ad libitum* and three of the rats were sacrificed every day for 8 days. The livers of all of these rats contained only the normal amount of fat.

*Effect of Nicotinamide on Liver Choline*—A preliminary statement has been made by du Vigneaud *et al.* (16) that the ingestion of glycocyamine inhibited the growth of rats and that this inhibition could be alleviated by the further addition of methionine or choline to the diets of the rats. While no experimental data were reported, the effect of choline in this instance stands in contradistinction to the effect of choline in nicotinamide-containing diets, if it be assumed that both inhibitions are due to deprivation of the methyl group. It has also been reported (17) that the addition of glycocyamine to an otherwise adequate diet produces fatty livers and simultaneously reduces the liver choline to values considerably below those obtained on a low protein, no choline diet. It, therefore, seemed desirable to ascertain the effects of nicotinamide on liver choline. For this purpose a preliminary experiment was performed in which three groups of five male rats each, weighing about 80 gm., were fed a 15 per cent casein, 35 per cent lard basal diet which was identical with that used by Stetten and Grail (17), except that the usual synthetic vitamin supplement was employed instead of yeast. A summary of this experiment is presented in Table VI which also contains some of the results of Stetten and Grail for comparison.

Liver choline was determined by the method of Jacobi, Baumann, and Meek (18). Glycocyamine, fed at such a level as to permit unimpaired growth, reduced liver choline to but 2 per cent of its normal value. In contrast, nicotinamide fed at a concentration sufficient to produce a more than 50 per cent inhibition of growth produced a mildly fatty liver but had no significant influence on choline concentration in the liver. It was felt, however, that these results could be better evaluated if there were also available the results of feeding nicotinamide and choline at several concentrations. Table VII summarizes such an experiment. Each group consisted of five male rats weighing between 50 and 60 gm. at the start of the experiment. The data are all expressed as the mean values for each group. The basal diet was identical with that used in the preliminary experiment

above. The values for liver choline were determined by a modification of the method of Jacobi, Baumann, and Meek (18) in which methanol was used in place of the alcohol-ether mixture for the initial fat extraction and

TABLE VI  
*Effect of Nicotinamide on Liver Choline*

Dietary choline	Dietary nicotinamide	Dietary glycyoamine	Weight change	Liver weight	Liver fat	Choline N
mg per kg.	mg per kg.	mg per kg.	gm. per day	per cent of body weight	per cent of wet weight	mg. per gm. liver
0.07	0.0	0.0	2.8	4.2	4.9	0.240
0.00	0.0	0.0	2.6	5.8	20.3	0.140
0.07	30.0	0.0	1.2	4.9	10.3	0.231

Stetten and Grail						
0.07	0.0	0.0	1.6	5.6	4.0	0.246
0.00	0.0	0.0	1.2	7.2	10.3	0.108
0.07	0.0	8.3	1.5	6.1	16.7	0.005

TABLE VII  
*Effect of Feeding Various Concentrations of Choline and Nicotinamide*

Group No.	Dietary choline	Dietary nicotinamide	Initial weight	Final weight	Weight change	Food ingested	Liver weight	Liver fatty acids	Choline N
	per cent of total	per cent of total	gm.	gm.	gm. per day	gm. per day	gm.	per cent of wet weight	mg. per gm. liver
1	0.0	0.0	55	79	2.3	9.2	4.65	16.2	0.170
2	0.0	0.25	55	69	1.4	7.5	4.06	19.7	0.179
3	0.0	0.5	57	69	1.2	6.8	4.20	12.6	0.193
4	0.0	1.0	54	59	0.5	6.3	2.85	7.3	0.214
5	0.0	2.0	55	53	-0.2	5.1	2.61	6.5	0.224
6	0.07	0.0	56	77	2.1	9.0	3.70	5.0	0.263
7	0.07	0.25	54	76	2.2	8.2	4.00	4.7	0.250
8	0.07	0.5	54	69	1.4	6.9	3.52	10.2	0.244
9	0.07	1.0	57	67	1.0	6.9	2.98	7.8	0.251
10	0.07	2.0	57	59	0.2	5.1	2.74	3.9	0.254
11	0.25	0.0	54	74	2.0	9.1	3.82	3.0	0.272
12	0.25	0.25	52	71	1.9	8.2	3.34	3.1	0.275
13	0.25	0.5	55	71	1.6	7.7	3.89	3.1	0.271
14	0.25	1.0	53	61	0.8	7.3	2.79	3.3	0.265
15	0.25	2.0	55	56	0.1	5.8	3.04	4.4	0.252

the alkaline digestion was carried out at 100°. These modifications have also been incorporated in the method of Engel (19).

When no dietary choline was present, the livers were quite fatty and the choline concentration was considerably below the normal, although growth



proceeded unimpaired. With increasing concentrations of nicotinamide growth was inhibited but the liver fat increased and then decreased in accordance with the growth principle stated previously. Simultaneously, the liver choline appeared actually to increase. This phenomenon is largely explained by the diluting effect of the fat in the livers of the rats that received little or no nicotinamide.

In the presence of a moderate amount of dietary choline, increasing levels of nicotinamide first produced a mild inhibition of growth and moderately fatty livers. At higher concentrations of nicotinamide, as growth was severely inhibited, the liver fat concentrations returned to normal. In no instance was there a significant decrease in the liver choline.

When a larger amount of dietary choline was used, nicotinamide produced the usual inhibition of growth. However, at no level of nicotinamide feeding were fatty livers induced and again no significant decrease in choline concentration was found.

The results of Groups 8 and 9 substantiate the findings of Jacobi and Baumann (20) in that here, too, it appears that normal concentrations of liver choline are not necessarily incompatible with fatty livers.

#### DISCUSSION

While this work was in progress, there appeared a preliminary report by Stekol (11) that nicotinic acid inhibited the growth of male rats on low casein diets. Further, it was stated that this inhibition could be alleviated by the administration of methionine and by choline together with homocystine or cystine but not by choline, cystine, or homocystine alone. The failure of nicotinic acid to inhibit seriously the growth of our rats under much the same conditions may be attributed to a quantitative strain difference, although we tested three strains of rats.

The inhibition of rat growth which results from the ingestion of nicotinamide may be ascribed to the deprivation of methyl groups by trigonelline synthesis, the toxicity of the unmethyated nicotinamide, or the toxicity of synthesized trigonelline, nicotinuric acid, etc. The latter possibility has been experimentally eliminated. No definitive experiment has been performed to test the other alternatives. Nevertheless, for the purposes of this discussion the first possibility will be assumed to be correct.

The findings presented here suggest that nicotinamide rather than nicotinic acid is the immediate precursor of trigonelline within the rat. Methionine or some derivative of methionine can act as the source of the necessary methyl group. The methyl groups of choline and betaine do not seem to be readily available to the rat for trigonelline synthesis. This would seem to argue against the operation of a "pool of labile methyl groups," at least in any quantitative fashion. However, both of these substances do

prevent the formation of the fatty livers associated with the ingestion of large amounts of nicotinic acid or moderate amounts of nicotinamide. This would suggest that the fatty livers induced in rats by methionine or choline deficiencies should be ascribed to a deficiency in choline or its derivatives rather than to a deficiency in "labile methyl groups" unless the latter be understood in a very strict and limited sense; *viz.*, choline precursors.

If homocysteine does arise from methionine in transmethylation reactions, dietary choline should be expected to contribute its methyl groups to the homocysteine resulting from the methylation of nicotinamide by the available dietary methionine and then effect the synthesis of more trigonelline from the newly synthesized methionine in the fashion demonstrated for dietary choline plus homocystine. The combination of dietary choline plus homocystine has been incontrovertibly demonstrated to yield tissue methionine (21). However, since choline does not stimulate growth in the presence of nicotinamide and a small amount of methionine (as dietary casein), it must follow that either homocysteine does not arise in methionine demethylation or that, while this does occur, the mechanism for resynthesis to available methionine is not efficient. The finding of Binkley and du Vigneaud (22) affords a mechanism for the latter possibility, suggesting that the path of methionine metabolism after demethylation may lead almost entirely to cystine formation if the diet contains suboptimal amounts of cystine. That the presence of large amounts of cystine may affect the existing equilibria in favor of the existence and remethylation of metabolically formed homocysteine is indicated by Stekol's finding (11) that the administration of choline plus cystine can prevent the growth inhibition produced in his rats by nicotinic acid.

While glycocyamine ingestion, at a level at which growth was still unimpaired, resulted in an almost complete disappearance of liver choline, no level of nicotinamide ingestion induced a significant decrease in the concentration of liver choline. The only obvious interpretation of these facts which is compatible with the other data contained herein is the direct methylation of glycocyamine by choline despite the *in vitro* findings of Borsook and Dubnoff (23).

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#### SUMMARY

1. The inclusion of 1 per cent of nicotinamide in a 10 per cent casein diet almost completely inhibited the growth of rats of both sexes. At the 1 per cent level nicotinic acid had no effect upon growth but did induce

fatty liver formation. Even 2 per cent of nicotinic acid had only a slight effect upon growth. This inhibition is believed to be due to the deprivation of the animal's supply of methyl groups because of trigonelline synthesis.

2. The inhibition of growth due to nicotinamide was prevented by the administration of methionine and by choline plus homocystine but not by choline, betaine, homocystine, or cystine alone. Fatty liver formation by nicotinamide and nicotinic acid was prevented by the feeding of methionine, choline, and betaine but was aggravated by feeding cystine or homocystine.

3. Trigonelline excretion was greater after the ingestion of nicotinamide than of nicotinic acid. This excretion was further increased by the administration of methionine and slightly increased by choline and betaine.

4. While the ingestion of sufficient nicotinamide to produce a growth inhibition of 50 to 75 per cent also resulted in fatty liver formation, when higher levels of nicotinamide were employed the growth inhibition was complete and only normal concentrations of liver fat were observed.

5. At no level of nicotinamide ingestion was a significant decrease in the concentration of liver choline observed.

6. The metabolic significance of these findings is discussed.

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## ISOLATION OF ANDROSTERONE SULFATE

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It has long been recognized that many of the steroids, including the androgens, are excreted in urine in conjugation with other substances which render them not only more water-soluble but also neutralize their physiological activity to a great extent.

In 1936 Cohen and Marrian (1) isolated estriol glucuronide, the conjugated form of estriol, from the urine of pregnant women and in 1936 Venning and Browne (2) reported the isolation of pregnanediol glucuronide from the same source. Schachter and Marrian in 1938 (3) isolated estrone sulfate from the urine of pregnant mares and in this paper we report the isolation of a conjugated androgen, androsterone sulfate, from the urine of a man suffering from an interstitial cell tumor of the testis with general metastases. Since this finding was reported (4), Munson, Gallagher, and Koch (5) have recently isolated a 17-ketosteroid sulfate in the form of a semicarbazone from normal men's urine which on hydrolysis yields dehydroisoandrosterone.

The first indication that the androgens might be excreted as conjugates was suggested by the observations of Funk, Harrow, and Lejwa in 1929 (6) that extraction of highly acidified male urine gave a better yield of the hormone promoting comb growth than extraction of neutral urine, and later Adler in 1934 (7) found that a physiologically inactive extract could be obtained from male urine with butanol. By subsequent heating with trichloroacetic acid the inactive extract could be converted into a potent substance which promoted the growth of the comb of the capon.

In the summer of 1941 through the courtesy of Professor P. Masson we were fortunate in being able to collect urine over several weeks from a patient with extensive metastases from an interstitial cell tumor of the testis. The history and the pathological findings of this case have been published by Professor Masson (8).

In 1932 a tumor of the left testis was removed from a 32 year-old laborer. For 9 years the man continued to work and was in perfect health. In May, 1941, he consulted a doctor, because his abdomen was enlarging and he found it difficult to bend when digging at work. On examination it was found that an enormous mass filled the abdomen and there was a tumor of one rib. The patient showed no sign of cachexia or of unusual hair growth or overdevelopment of the secondary sex organs; his strength

was unimpaired. 6 weeks later he died suddenly. At autopsy the most striking finding was that the liver weighed 7.5 kilos and was filled with spherical metastases. The histological examination of these metastases showed the same structure as the original tumor.

It is of interest to note that neither this patient nor the one previously described by Masson and Sencert (9) ever became cachectic in spite of extensive metastases and both preserved to the end their strength and muscular system. One may speculate as to whether this is related to the production of large amounts of androgenic substances by these tumors which maintain the individual in positive nitrogen balance. Assays were carried out on urine and blood. The urinary estrogens and gonadotropins were only moderately increased, 113 and 110 mouse units respectively, while the 17-ketosteroids were markedly raised, the average excretion being 1015 mg. per 24 hours. The serum 17-ketosteroid was elevated 16 mg. per cent.

A partially purified extract of the hydrolyzed urine containing 50 per cent 17-ketosteroids by weight was sent to Dr. Koch for assay on the capon, and the androgenic activity of this mixture was found to represent a content of 12 to 18 per cent androsterone or 48 to 72 per cent dehydroisoandrosterone. This high androgen content in relation to the 17-ketosteroid led us to investigate the nature of the conjugated androgen present in the urine. The free steroids are also being studied and the findings will be reported at a later date.

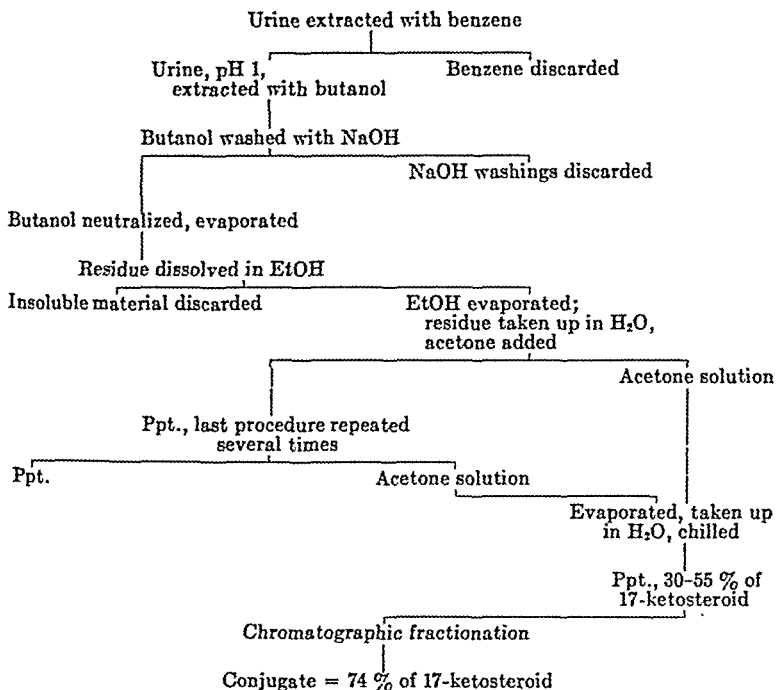
#### EXPERIMENTAL

Melting points were determined on a Fisher-Johns micro melting point apparatus and are uncorrected. Microanalyses were kindly carried out by Mrs. Dorothy Jewitt of Ayerst, McKenna and Harrison, Ltd.

The 17-ketosteroids were determined colorimetrically by the Holtorff and Koch modification (10) of the Zimmermann reaction. This assay served as a guide in following the various stages of purification. At first the assay was carried out after acid hydrolysis; it was later found, however, that as the various fractions were purified (content at least 30 per cent 17-ketosteroids) the Zimmermann reaction could be applied directly to the unhydrolyzed material.

The method of isolation depends upon solvent partition, as shown in the accompanying flow sheet.

*Extraction and Purification*—38 liters of urine were collected which contained 18.3 gm. of 17-ketosteroid. The urine was extracted with benzene to remove all the free steroids (3.5 gm.), leaving 14.8 gm. of the conjugated 17-ketosteroid. The urine was then acidified to pH 1 with HCl and extracted seven times with butanol. At this pH, 95 per cent



of the conjugated 17-ketosteroids pass into the butanol. The butanol extract was neutralized with 20 per cent NaOH and then washed four times with 2 *N* NaOH. Considerable purification was effected by this washing, although 14 per cent of the conjugated 17-ketosteroids was also removed from the butanol by this procedure. The butanol extract was then neutralized with HCl and evaporated to dryness under reduced pressure. 2.1 gm. of 17-ketosteroid were removed by the NaOH washing. The residue at this stage weighed 104.3 gm. and contained 12.7 gm. of 17-ketosteroid. It was dissolved in hot ethanol, centrifuged, and the insoluble material was discarded. The ethanol was evaporated to dryness under reduced pressure. The residue was dissolved in 30 cc. of water and to the solution were added 400 cc. of acetone. The supernatant fluid was poured off and this procedure was repeated several times on the gummy precipitate with smaller amounts of water each time. All the aqueous acetone solutions were combined and evaporated to dryness under reduced pressure. A small volume of water was added to dissolve the gummy residue. This aqueous solution was evaporated under reduced pressure until cloudiness appeared. It was then transferred to a centrifuge

tube, chilled, and centrifuged. A light brown substance was precipitated. This material was dissolved in a small volume of water and the last procedure was repeated. An almost colorless gummy material was obtained. When this material was dried under reduced pressure, an amorphous substance containing 30 to 50 per cent of 17-ketosteroid was obtained.

*Fractionation of Conjugated 17-Ketosteroid*—Small fractions of the conjugate (100 to 200 mg.) were dissolved in a minimal amount of ethanol and acetone was added until the mixture was approximately 80 per cent acetone. It was then adsorbed on a column of Merck's activated alumina prepared according to the directions of Brockmann. Fractional elution was carried out with pure acetone at first, then with mixtures of this solvent and ethanol (10, 30, and 40 per cent ethanol-acetone). A relatively pure fraction of conjugated 17-ketosteroid as judged by the Zimmermann assay was eluted by the 40 per cent ethanol-acetone mixture. The fractions containing the highest content of 17-ketosteroid were united and readsorbed on alumina. By repeated chromatographic fractionations a colorless substance was obtained which contained 70 to 74 per cent of 17-ketosteroid, the theoretical yield from a compound having the structure of sodium androsterone sulfate being 74 per cent 17-ketosteroid.

The compound so obtained was amorphous and considerable difficulty was encountered in trying to obtain crystalline fractions from either ethanolic or aqueous acetone solutions, since gel formation usually occurred. The solid material obtained when these gels were dried *in vacuo* contained 74 per cent 17-ketosteroid and melted at 153°. Crystalline material was obtained by allowing an ethanolic solution of the compound to stand at room temperature for several days. From the original 14.8 gm. of conjugated 17-ketosteroid, over 2 gm. of amorphous conjugate containing 74 per cent 17-ketosteroid were obtained; of this amount 600 mg. were in crystalline form.

*Characterization of Conjugated 17-Ketosteroid*—Two different forms of crystals were obtained, depending upon the solvent from which they were obtained. They were both needle-like in character and could easily be converted one into the other by changing the solvent.

The crystals obtained from acetone containing a few drops of water melted sharply at 144°, without decomposition. Those obtained from an ethanolic solution melted at approximately 190°; however, a definite melting point could not be obtained, because decomposition occurred with the probable formation of  $\text{Na}_2\text{SO}_4$ . The crystals would frequently become opaque without any visible signs of melting. The analysis of the two forms showed that the difference was due to water of crystallization.

The conjugate is readily soluble in water, methanol, and glacial acetic acid, less soluble in ethanol, and only sparingly soluble in anhydrous

acetone. It is insoluble in ether, benzene, and chloroform. Qualitative tests were positive for Na and S, the S being present as inorganic sulfate. The conjugate gave a negative Tollens test for glucuronic acid. It was saturated, as no iodine was taken up in the Rosenmund-Kuhnnehn method; the Rosenheim test was also negative. The fact that this compound gives a positive Zimmermann test with maximum absorption at 520  $m\mu$  indicates that it contains a free ketone group at C<sub>17</sub>.

Quantitative analysis of the two crystalline forms shows the values given in Table I. These values agree with the theoretical values calculated for a compound having the structure of sodium androsterone sulfate. The crystalline form melting at 144° contained no water of crystallization, while the form melting at 190° contained 1 molecule of water of crystallization. The latter was dried for 3 hours at 80° *in vacuo* over P<sub>2</sub>O<sub>5</sub> without loss in weight.

TABLE I  
*Analysis of Sodium Androsterone Sulfate*

	(C <sub>19</sub> H <sub>27</sub> O <sub>2</sub> )SO <sub>3</sub> Na		(C <sub>19</sub> H <sub>27</sub> O <sub>2</sub> )SO <sub>3</sub> Na(H <sub>2</sub> O)	
	Calculated	Found, m.p. 144°	Calculated	Found, m.p. 190°
C.....	58.16	58.16	55.85	55.82
H.....	7.42	7.40	7.57	7.79
Na.....	5.85	5.80	5.61	5.61
S.....			7.80	7.79

*Hydrolysis of Conjugated 17-Ketosteroid*—The proof that this conjugated 17-ketosteroid is sodium androsterone sulfate is supplied by the identification of the steroids produced on hydrolysis. The method chosen was that of simultaneous hydrolysis and extraction of Dingemans, Borchardt, and Laqueur (11) with CCl<sub>4</sub> as the solvent. This procedure has been considered to be the one least likely to cause alteration in the original steroid. The acid content was varied and it was found that the maximum yield of free 17-ketosteroid was obtained when the mixture contained 8 to 10 per cent HCl. Even with this mild form of hydrolysis considerable destruction occurred. Four lots of pure sodium androsterone sulfate (80 to 105 mg.) were hydrolyzed by this method. The sodium androsterone sulfate was dissolved in 100 cc. of H<sub>2</sub>O containing 8 to 10 cc. of HCl and 50 cc. of CCl<sub>4</sub> were added. The mixture was refluxed for 6 hours, the CCl<sub>4</sub> being changed at  $\frac{1}{2}$ , 1, 2, and 4 hour periods. The CCl<sub>4</sub> was washed with 2 N NaOH and then with H<sub>2</sub>O until the washings were neutral. The CCl<sub>4</sub> extract was taken to dryness and weighed. The amount of hydrolyzed material by weight represented 50 to 65 per cent of the original



17-ketosteroid present in the compound. When the 17-ketosteroid content of these fractions was determined, the amount of 17-ketosteroids recovered was found to be even lower. However, when crude fractions of the conjugate containing 4 to 28 per cent 17-ketosteroids were hydrolyzed by the same method, approximately 80 per cent of the free 17-ketosteroids could be recovered, as shown in Table II. This would suggest that compounds present in the urine act in some way as a protective mechanism, to prevent destruction of the 17-ketosteroids on hydrolysis, and consequently the loss of these compounds may not be as great when they are hydrolyzed in urine of which the content of 17-ketosteroids is relatively small compared to that of other urinary compounds.

TABLE II  
*Hydrolysis of Pure and Crude Fractions of Conjugate*

Weight of sample	17-Ketosteroid, unhydrolyzed	17-Ketosteroid, after hydrolysis	Recovery	Method of hydrolysis*
mg.	mg.	mg.	per cent	
10	7.4	4.2	56.7	A
10	7.4	4.1	55.4	B
90 (Crystalline)	63.3	30.6	48.3	"
80 "	56.3	22.8	40.5	"
47 (Crude)	1.7	1.2	70.6	A
43 "	5.1	4.1	80.2	"
75 "	15.2	12.4	81.5	"
200 "	57.3	50.5	88.2	" (10%)
200 "	57.3	46.9	82.0	B (10%)
200 "	57.3	47.5	82.8	C
1000 "	220	180	82.0	A (15%)
1000 "	220	165	75.0	B

\* Method A, boiling with 8 per cent HCl 15 minutes, extraction with benzene (17); Method B, simultaneous hydrolysis and extraction with CCl<sub>4</sub> and 8 to 10 per cent HCl 5 to 6 hours (18); Method C, boiling with 4 per cent HCl 50 minutes (19).

*Fractionation of Hydrolyzed Material*—The hydrolyzed material was dissolved in benzene-pentane mixture and passed through a column of Brockmann's alumina. The column was eluted with benzene-pentane mixtures and then with benzene, as indicated in Table III. By this method two crystalline compounds were isolated from the hydrolyzed material. The compound obtained from the first eluates melted at 99–101°.

On recrystallization from aqueous methanol and subsequent sublimation, (90–100° at 0.001 mm.) colorless rectangular plates were obtained, which melted at 105.5–107°. A mixed melting point determination with an authentic urinary sample of androstenone-17 kindly supplied by Dr. Hirschmann showed no depression.

*Oxime of Androstenone*—The oxime of androstenone-17 was prepared in the following manner: 9 mg. of androstenone-17 and 25 mg. of hydroxylamine hydrochloride were dissolved in 3 cc. of ethanol and 5 mg. of sodium acetate dissolved in 1 cc. of H<sub>2</sub>O were added. The ethanol was evaporated *in vacuo* and the crystalline material which separated out was recrystallized three times from aqueous ethanol. The melting point of the oxime was 153–154° and there was no depression when the material was mixed with an authentic sample of androstenone oxime kindly supplied by Dr. Pearlman.

TABLE III  
*Fractionation of Hydrolyzed Material*

10 cc. of material were used in each instance.

Fraction No.	Eluent	Eluate	M.p.	Compounds isolated
		mg.	°C.	
1	50% benzene-pentane	0		
2	50% "	2.3	99–101	
3	50% "	5.2	102–104	
4	50% "	2.6		Androstenone-17, m.p.
5	50% "	1.3		104–105°
6	50% "	0.6		
7	50% "	0.4	Oil	
8	50% "	0		
9	60% "	0		
10	60% "	0		
11	100% benzene	19.5	149–159	
12	100% "	10.5		Androsterone, 182–184°
13	100% "	2.0		
14	100% "	0		

*Analysis*—C<sub>19</sub>H<sub>28</sub>NOH. Calculated. C 79.44, H 10.13, iodine No. 89  
Found. " 79.43, " 10.24, " " 94

The C, H, and N analyses agree with the theoretical values for the oxime of androstenone-17. The iodine value of the oxime was 94, indicating the presence of one double bond. These findings identify the original compound as androstenone-17. The position of the double bond has not been definitely established. The discrepancies between the melting points of androstenone-17 obtained by removal of hydrogen chloride from  $\alpha$ -3-chloroandrostanone-17 and the androstenone-17 obtained from other sources suggest that these substances may not be homogeneous but mixtures of  $\Delta$ -2- and  $\Delta$ -3-androstenone. This has been fully discussed by Hirschmann (12).

The second crystalline fraction was eluted from the column with benzene,

m.p. 145–159°. On repeated crystallization from dilute methanol, plates were obtained which melted at 182–184° and showed no depression when mixed with authentic androsterone.

*Androsterone Acetate*—The acetate was prepared by heating a sample of the compound for 1 hour at 100° with acetic anhydride. The mixture was diluted with water and extracted with ether, washed with NaOH, and then with water. The ether was evaporated and the androsterone acetate was recrystallized several times from aqueous ethanol. It melted at 164–165° and showed no depression with authentic androsterone acetate.

*Analysis*— $C_{19}H_{28}O \cdot C_2H_4O_2$ . Calculated. C 75.90, H 9.63  
Found. " 75.79, " 9.48

These findings identify the second crystalline compound as androsterone.

*Sodium Androsterone Sulfate Semicarbazone*—The semicarbazone of the conjugate was also prepared. 30 mg. of conjugate were dissolved in a small volume of water, 30 mg. of semicarbazide hydrochloride and 30 mg. of sodium acetate were added, and the mixture was gently heated for 1 hour at 50° and then allowed to stand at room temperature for several days. The crystals obtained melted at 245° and were only sparingly soluble in hot water.

#### DISCUSSION

The evidence furnished by the analysis, the proportion of 17-ketosteroid in the conjugate, and the identification of the two 17-ketosteroids produced on hydrolysis as androsterone and androstenone-17 all point to the fact that this conjugate is androsterone sulfate.

The relative amounts of androstenone-17 and androsterone derived from the hydrolysis of different lots of pure conjugate varied considerably, even though the conditions of hydrolysis were similar. As seen from Table IV, the early eluates which contained the androstenone-17 fraction varied from 13.7 to 25.2 per cent of the original sample, whereas the late eluates containing the androsterone fraction varied from 8.2 to 30.5 per cent. The 17-ketosteroid content of the three fractions of Conjugate C is given. The fact that a saturated 17-ketosteroid sulfate yields on hydrolysis a saturated hydroxy compound, androsterone, and a non-hydroxy, unsaturated one, androstenone-17, indicates that the  $SO_4$  group can be split off in two different ways, as outlined in Fig. 1. The sulfate group may be split off with the formation of the original ketosteroid androsterone or  $H_2SO_4$  may be removed from the molecule with the resulting formation of a double bond between  $C_2$  and  $C_3$  or between  $C_3$  and  $C_4$  (Fig. 1).

The formation of this non-hydroxy 17-ketosteroid is of interest with regard to recent work on the isolation of steroids from normal and patho-

logical urine. The question has arisen whether the non-hydroxy 17-ketosteroids such as androstenone-17,  $\Delta^3,^5$ -androstadienone-17, and 3-chloro- $\Delta^5$ -androstenone-17 are products of intermediary metabolism or artifacts produced by the action of acid hydrolysis on the free or conjugated steroid. Butenandt *et al.* (13) have shown that 3-chloro- $\Delta^5$ -androstenone-17 is formed by the action of hydrochloric acid on dehydroisoandrosterone during the process of hydrolysis and recently we have hydrolyzed synthetic dehydroisoandrosterone sulfate kindly prepared for us by Dr. Lieberman

TABLE IV  
Recovery Following Hydrolysis of Pure Conjugate

	Conjugate A, 105 mg., recovery weight	Conjugate B, 90 mg., recovery weight	Conjugate C, 90 mg.	
			Weight	17-Ketosteroid
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Hydrolyzed material.....	48.8	40.0	44.5	33.2
Early eluates .....	13.7	20.5	25.2	23.8
Late " .....	30.5	11.1	8.2	7.4

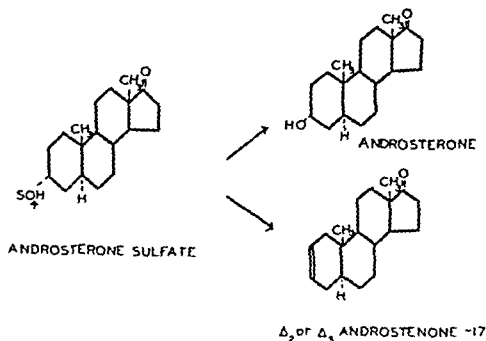


FIG. 1. Hydrolysis of androsterone sulfate

and have found that it yields on hydrochloric acid hydrolysis equal quantities of dehydroisoandrosterone and 3-chloro- $\Delta^5$ -androstenone-17. That  $\Delta^3,^5$ -androstadienone-17 may also be formed in the process of hydrolysis from dehydroisoandrosterone is suggested by the findings of Dobriner *et al.* (14) and by Pincus and Pearlman (15). By analogy from our findings on the hydrolysis of androsterone sulfate,  $\Delta^3,^5$ -androstadienone-17 might easily arise from the hydrolysis of dehydroisoandrosterone sulfate under certain conditions. Hirschmann (12) was the first to isolate androstenone-17 from human urine. He obtained this non-hydroxy steroid from the urine of ovariectomized women and suggested that this compound might be

formed by the hydrolysis of a conjugated form of androsterone. Our findings furnish proof for his theory. Pearlman (16) also isolated this compound from the urine of normal females and of cancerous males and more recently Dobriner and coworkers (14) have isolated this compound from the urine of two normal individuals and three with adrenal hyperplasia. The present methods of hydrolysis are far from satisfactory and, as we have shown, considerable destruction and alteration of the original steroid occurs even under the mildest conditions. The variability in the yield of the two steroids obtained from the hydrolysis of androsterone sulfate under similar conditions should make one cautious in interpreting the significance of the proportion of these compounds in urine from various sources. The isolation of the conjugated form of androsterone and dehydroisoandrosterone now offers a means for studying and improving the conditions of hydrolysis for these compounds.

A bioassay carried out on the capon revealed that sodium androsterone sulfate was only weakly androgenic; i.e., 1 to 2 per cent as active as androsterone. After acid hydrolysis the activity rose to 16 per cent of androsterone. These assays must be considered as qualitative, however, as only two capons were available for each test.

#### SUMMARY

1. A crystalline conjugated androgen sodium androsterone sulfate has been isolated from the urine of a man suffering from an interstitial cell tumor of the testis.

2. This compound exists in two crystalline forms, one melting at 144° and a second containing 1 molecule of water of crystallization and melting at approximately 190° with decomposition.

3. This compound is soluble in water and alcohol, insoluble in ether and benzene.

4. Sodium androsterone sulfate is only weakly androgenic.

5. On hydrolysis with acid it yields androsterone and a non-hydroxy unsaturated compound, androstenone-17.

6. The relative amounts of these two steroids vary under similar conditions of hydrolysis.

One of us (M. M. H.) is indebted to the Banting Research Foundation for a personal grant.

We wish to thank Dr. Koch for his kindness in carrying out the bioassays.

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# CANINE CYSTINURIA. THE CYSTINE OUTPUT ON AN ARACHIN DIET\*

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In previous work Hess and Sullivan (1) have reported on the excretion of cystine in the urine of two cystinuric, male Irish terriers following the administration of diets containing various levels of casein and also upon the excretion of extra urinary cystine when either methionine or cysteine was added to these diets. Casein contains approximately 0.3 per cent cystine and about 10 times as much methionine. If the urinary cystine in cystinuria is derived mainly from the ingested methionine rather than from the cystine, it would be of interest to substitute for the casein in the diet a protein relatively rich in cystine and poor in methionine, a situation just the reverse of that found in the casein. A search of the literature revealed that the only protein approximating these conditions is arachin, which contains 1.29 per cent cystine (Sullivan and Hess (2)) and 0.54 per cent methionine (Baernstein (3)). Substitution of arachin for casein at similar levels as were previously employed and the subsequent feeding of both methionine and cysteine should reveal information of value in the relationship of diet and amino acids in canine cystinuria.

## EXPERIMENTAL

The arachin was prepared from blanched, raw peanuts by the method of Johns and Jones (4). The arachin contained 1.26 per cent cystine by the Sullivan method (2) and 0.41 per cent methionine by the McCarthy-Sullivan method (5) on the ash- and moisture-free basis. The diet was the same as that previously employed (1) except that arachin was substituted for casein at 10 and 25 per cent levels. The amino acids fed, *L*-methionine and cysteine hydrochloride, were analytically pure. The *L*-methionine was isolated from casein. Each dog received approximately 150 gm. of diet per day. The weighed amount of each amino acid was intimately mixed with the diet each day.

The same two cystinuric dogs, Nos. 32-T and 38-U, were employed as with the casein experiments. The daily collection and analysis of the urine specimens were carried out as in the previous study. Table I gives

\* A preliminary report of this work was presented before the meetings of the American Society of Biological Chemists held at Boston, March 31 to April 4, 1942 (*Federation Proc.*, 1, pt. 2, 115 (1942)).



the average daily excretion of the principal substances determined in the urines of both dogs when the basal diets containing 10 and 25 per cent

TABLE I

*Average Daily Urinary Excretions during Control Periods on Two Protein Levels*

Arachin level	Dog No.	Urine			
		Cystine	Total S	Neutral S	Nitrogen
<i>per cent</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
10	32-T	0.007	0.140	0.010	1.98
	38-U	0.008	0.137	0.039	1.96
25	32-T	0.031	0.215	0.070	4.90
	38-U	0.032	0.209	0.066	4.80

TABLE II

*Total Urinary Excretion for 4 Day Period during Ingestion of Amino Acids upon Two Protein Levels*

	Dog No.	2.0 gm. methionine		2.6 gm. cysteine HCl	
		10 per cent arachin level	25 per cent arachin level	10 per cent arachin level	25 per cent arachin level
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Cystine	32-T	0.059	0.210	0.146	0.408
	38-U	0.058	0.227	0.125	0.361
Nitrogen	32-T	8.05	20.10	8.02	20.47
	38-U	8.26	19.83	8.01	19.70
Total S	32-T	0.897	1.151	0.791	1.326
	38-U	0.741	1.143	0.911	1.126
Neutral S	32-T	0.183	0.360	0.206	0.612
	38-U	0.172	0.285	0.162	1.374

TABLE III

*Urinary Excretion of Extra Cystine Following Ingestion of Amino Acids (4 Day Period)*

Arachin level	Dog No.	Methionine	Cysteine
<i>per cent</i>		<i>gm.</i>	<i>gm.</i>
10	32-T	0.031 (110)*	0.118 (421)*
10	38-U	0.026 (81)	0.093 (281)
25	32-T	0.074 (54)	0.272 (200)
25	38-U	0.099 (77)	0.232 (181)

\* The figures in parentheses are the percentage increases in cystine excreted above the control.

arachin were fed. The average value in each case is based upon a number of determinations made both before and after the feeding of the various

amino acids. The values upon which the averages are based are, in all cases, close together. Since arachin is known to be a deficient protein, the animals were not kept on the diet more than 10 days at a time.

In Tables II and III data are presented on the effect of feeding methionine and cysteine hydrochloride on the same urinary constituents. Of the amount of the two amino acids indicated, one-half was fed daily for 2 successive days. The urine was collected for these 2 days and also for the following 2 days, since a large number of data showed that it required this period to insure a return to normal levels. Since we have never found any extra cystine following the administration of cystine, this amino acid was omitted from the experiment.

#### DISCUSSION

As judged by the urinary cystine, there is a marked difference in the effect of feeding the same levels of casein and arachin. The daily cystine excretion on the 10 per cent arachin diet is the same as on the 5 per cent casein diet, while the cystine excretion on the 25 per cent arachin diet is slightly less than the cystine excretion on the 10 per cent casein diet. The difference between the results with casein and arachin cannot be due to indigestibility and poor absorption of the arachin, since the total nitrogen of the urine on the 10 and 25 per cent arachin diets does not differ much from that on the corresponding casein diets. On the 10 per cent arachin diet the daily cystine output is one-fifth that on the 10 per cent casein diet and on the 25 per cent arachin diet the cystine output is about half that on the 25 per cent casein diet, while the methionine content of both arachin diets is one-seventh that of the corresponding casein diets. Some other factor than the mere percentage content of methionine is involved.

As with casein, increased amounts of arachin in the diet gave a larger output of cystine in the control periods and as with casein the feeding of cysteine and methionine gave less extra cystine excretion on the high protein diet than on the lower protein diet. In the casein diet both methionine and cysteine exert percentagely their most marked effect upon cystine excretion at the 5 per cent level and in the arachin diet at the 10 per cent level. In total output, however, the greatest excretion of extra cystine after methionine and cysteine were fed was on the 10 per cent casein and on the 25 per cent arachin diets. At the 25 per cent arachin level the ingestion of 2 gm. of methionine produced an increase in cystine excretion in both dogs that is greater than that produced by the similar supplement at the 25 per cent casein level. Thus on the arachin diet the average increase of extra cystine was 87 mg., while for the casein diet it was only 21 mg. The excretion of 210 and 227 mg. of total cystine during the 4 day period on the 25 per cent arachin diet plus methionine is significant when

compared with the excretion of 136 and 128 mg. of cystine during the 4 day control period. The values for the control days never varied more than 5 mg. from the mean for Dog 32-T and 1 mg. for Dog 38-U.

In the casein experiments the addition of 2 gm. of methionine to the diet led to the excretion of more extra cystine than did the addition of 2 gm. of cysteine. In the arachin feeding, on the other hand, cysteine caused far more extra cystine in the urine than did methionine. Thus on the 10 and 25 per cent arachin diets the addition of 2 gm. of methionine caused an average extra cystine excretion respectively of 29 and 87 mg., while 2 gm. of cysteine caused an average extra cystine excretion of 106 and 252 mg. respectively.

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# THE PHOTOCHEMICAL SPECTRUM OF CYTOCHROME OXIDASE\*

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(Received for publication, September 12, 1942)

The respiratory ferment in living microorganisms has been shown to exhibit a photochemical absorption spectrum typical for pheohemin compounds (2-4). The two relative absorption coefficients obtained by Warburg and Negelein (5) for rat retina by determining the effect of light in decreasing aerobic glycolysis of CO-treated retinas have been shown to represent points on the spectrum of the Pasteur enzyme (6). Generally identified with the respiratory ferment in animal tissues is *cytochrome oxidase*, which catalyzes the oxidation of cytochrome *c* (7).

For an investigation of the spectrum of cytochrome oxidase in mammalian tissue, phosphate extracts of rat heart muscle were selected as the source of enzyme. When succinate was employed as substrate, the addition of cytochrome *c* failed to increase the oxygen consumption; thus, the extracts contained an excess of cytochrome *c*. Although the over-all reaction consisted in the oxidation of succinate to fumarate, there is ample evidence to show that this reaction is mediated by the cytochrome-cytochrome oxidase system (8). Keilin and Hartree (7) have shown that CO is a strong inhibitor of cytochrome oxidase. This inhibition is easily relieved by light. As the heart muscle extracts take up O<sub>2</sub> vigorously in the presence of succinate at temperatures as low as 10°, this system lends itself to an analysis by the photochemical technique.

## EXPERIMENTAL

*Enzyme System*—Hearts were removed from freshly killed adult rats, and washed free from blood. Either extracts were prepared immediately, or the washed hearts were frozen and extracts made as needed. After being cut into small pieces, the heart muscle was ground in the presence of sand, with 10 times its weight of 0.1 M sodium phosphate buffer at pH 7.3. After centrifugation at 3000 R.P.M. for 15 minutes, the supernatant suspension was used as the enzyme preparation. 2 cc. of the suspension were placed

\* This work was carried out by the author during the tenure of a Finney-Howell Research Foundation Fellowship (1939-41). It was aided by a grant made to Dr. Kurt G. Stern by the Jane Coffin Childs Memorial Fund for Medical Research. A preliminary note on this work has been published (1).

in one of the cylindrical vessels (volume, 20.2 cc.) of the differential manometer and 0.2 cc. of a neutralized succinate solution added, so that the final concentration of succinate was 0.01 M. The compensation vessel of the manometer contained 2.2 cc. of the buffer-succinate solution. The tem-

TABLE I

*Photochemical Effect at 436 mμ Versus That at 407 mμ on CO Inhibition of Cytochrome Oxidase in Extract of Rat Heart Muscle*

Time	Light			Manometer deflections $\left(\frac{\Delta p}{5 \text{ min.}}\right)$	Manometer deflections in dark by interpolation $\left(\frac{\Delta p}{5 \text{ min.}}\right)$	Photoactivity $\left(\frac{\Delta p}{(5 \text{ min.})} - \left(\frac{\Delta p}{5 \text{ min.}}\right)_d\right) \times 100$
	Wave-length	Source	Intensity			
(1)	(2)	(3)	(4)	(5)	(6)	(7)
min.	mμ		gm. calorie per sq. cm. per min.	mm.	mm.	per cent
5				3.84		
10				3.92		
15	436	Hg arc	$0.50 \times 10^{-4}$			
20	436			5.15	3.86	33.4
25	436			4.97	3.85	29.1
30						
35				3.83		
40	407	Sr-carbon arc	$5.7 \times 10^{-4}$			
45	407			5.08	3.82	33.0
50	407			5.17	3.81	35.7
55						
60				3.80		
65	436	Hg arc	$0.34 \times 10^{-4}$			
70	436			4.57	3.78	20.9
75	436			4.53	3.77	20.2
80						
85				3.75		
90				3.75		

perature of the thermostat was 10°. The rate of rotation of the vessels was 500 R.P.M.

It was found that under these conditions neither the presence of KOH in an inner well, nor the addition of more succinate, nor the addition of cytochrome *c* influenced the manometric readings. Whereas replacement of the air in the manometric vessels with a mixture of 95 per cent N<sub>2</sub> and

5 per cent  $O_2$  had no effect on the activity of the enzyme, an atmosphere of 95 per cent  $CO$  and 5 per cent  $O_2$  resulted in a 75 per cent decrease in the  $O_2$  uptake.

*Determination of Relative Absorption Coefficients ( $\beta_\lambda/\beta_{436}$ )*—The arrangement of the photochemical apparatus and the method of charting photochemical absorption spectra have already been described (3, 6). In the experiments described in the present paper, the photochemical effect consisted of an increase in the  $O_2$  consumption when heart muscle extracts, in the presence of succinate and a gas phase of 95 per cent  $CO$  and 5 per cent  $O_2$ , were subjected to strong monochromatic illumination.

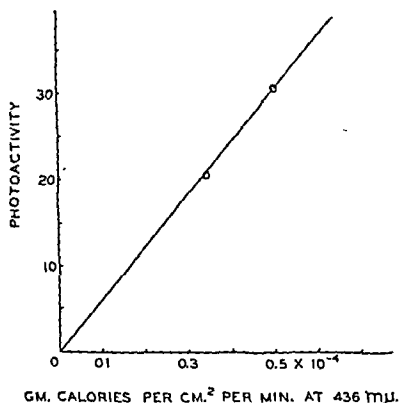


FIG. 1

FIG. 1. Photoactivity curve at 436  $m\mu$ .

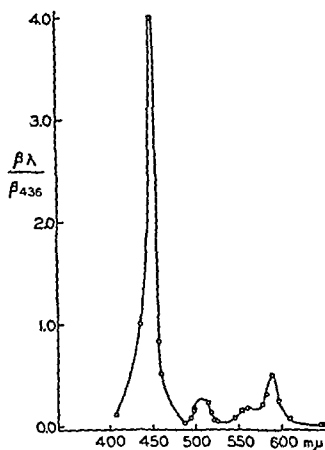


FIG. 2

FIG. 2. Relative photochemical absorption spectrum of the  $CO$  compound of cytochrome oxidase from rat heart muscle.

The details of the method for determining the  $\beta_\lambda/\beta_{436}$  values for cytochrome oxidase follow those already given in a previous paper of this series (4). The data for a typical experiment are presented in Table I. In Column 6 of Table I are the interpolated values for the  $O_2$  uptake in the dark at the times when actually the  $O_2$  uptake values in the light were being measured. With the aid of these figures, the photoactivity values

$$\frac{(\Delta p/\Delta t)_l - (\Delta p/\Delta t)_d}{(\Delta p/\Delta t)_d} \times 100$$

may be calculated (Column 7).

In Fig. 1 the photoactivity at 436  $m\mu$  is plotted against the intensity ( $i$ ) at 436  $m\mu$ . From this curve it is found that an intensity of  $5.7 \times 10^{-4}$  gm. calorie per sq. cm. per minute at 407  $m\mu$  which exerts a photoactivity of 34.4 per cent is equivalent to 0.51 gm. calorie at 436  $m\mu$ . The relative absorption coefficient then may be arrived at according to the following equation.

$$\frac{\beta_{427}}{\beta_{417}} = \frac{i_{427} \times 436}{i_{417} \times 407} = \frac{0.51 \times 10^{-4} \times 436}{5.7 \times 10^{-4} \times 407} = 0.101$$

Four experimental determinations at 407  $m\mu$  yielded an average value of 0.11 for  $\beta_{407}/\beta_{436}$ .

TABLE II  
Relative Absorption Coefficients of Cytochrome Oxidase

Wave-length	Light source	$\frac{\beta_{\lambda}}{\beta_{417}}$	Wave-length	Light source	$\frac{\beta_{\lambda}}{\beta_{417}}$
$m\mu$			$m\mu$		
407	Sr	0.11	522	Cu	0.06
436	Hg	1.00	525	Sr	0.05
430-470	Cu	1.50	516	Hg	0.08
450	"	4.0*	553	Mg	0.15
457	Mg	0.83	560	Ca	0.18
460	Li	0.51	578	Hg	0.22
487	Sr	0.02	582	Sr	0.31
494	Mg	0.08	589	Na	0.50
497	Sr	0.16	597	Sr	0.25
497	Li	0.15	610	Li	0.07
515	Cu	0.22	640-650	Ca	0.005
517	Mg	0.13	640-655	Sr	0.00

\* Calculated; for the method, see Stern and Melnick (6).

### Results

The relative absorption coefficients, as determined in the manner just described for twenty-three wave-lengths in the visible region of the spectrum, 407 to 655  $m\mu$ , are presented in Table II. The values, when plotted against wave-length, yield the relative photochemical CO spectrum of cytochrome oxidase in the extract of rat heart muscle (Fig. 2).

As in the previous studies (4, 6), no source of intense monochromatic radiation was available at the very peak of the main absorption band. By application of the procedure already reported, the approximate height of the  $\gamma$ -band was determined, and the maximum located at 450  $m\mu$ .

### DISCUSSION

*Relationship of Cytochrome Oxidase to Respiratory Ferment of Microorganisms*—From these data it appears that cytochrome oxidase from a

mammalian source, like the respiratory ferment in yeast and in bacteria, exhibits a spectrum characteristic of pheohemin compounds. There is a steep  $\gamma$ -band in the blue at 450  $m\mu$  and two secondary maxima, the  $\beta$ -band in the blue-green at 510  $m\mu$ , and the  $\alpha$ -band in the yellow at 589  $m\mu$ . The thermolability of the enzyme suggests that the hemin grouping is combined with a protein. In spite of the similarity of these enzymes, there exist significant differences in details to indicate that they are not identical. Thus, the main absorption band is at 450  $m\mu$  in the instance of the enzyme of heart muscle, and at 430  $m\mu$  for that in acetic acid bacteria and in yeast (2-4).

*Identity of Cytochrome  $a_3$  and Cytochrome Oxidase*—The possible identity of cytochrome  $a_3$  and cytochrome oxidase has been discussed at length by Keilin and Hartree (8). When heart muscle preparations are reduced by succinate, these authors observed that  $\alpha$ -bands of the cytochromes are present at 605, 564, and 550  $m\mu$ , and  $\gamma$ -bands at 448, 432, and 415  $m\mu$ . Upon treatment of the reduced enzyme preparation with CO, the following changes occur: (1) Two new, faint bands appear, an  $\alpha$ -band at 590  $m\mu$  and a  $\gamma$ -band at 452  $m\mu$ ; (2) the strong band at 448  $m\mu$  disappears; and (3) the band at 432  $m\mu$  becomes stronger. In commenting on the spectra of cytochromes  $a$  and  $a_3$  in the reduced, CO-treated enzyme preparations, Keilin and Hartree make the following assignments: 605  $m\mu$  to the  $\alpha$ -band of cytochrome  $a$ , 590  $m\mu$  to the  $\alpha$ -band of cytochrome  $a_3$ , 452  $m\mu$  to the  $\gamma$ -band of cytochrome  $a$ , 432  $m\mu$  to the  $\gamma$ -band of cytochrome  $a_3$ . From the experiments reported in this paper, the case for the identity of cytochrome  $a_3$  and cytochrome oxidase would be stronger if the two new bands which appear in the presence of CO are both assigned to cytochrome  $a_3$ . The results would then fit in well with those obtained by the photochemical technique, the  $\alpha$ -band at 589  $m\mu$  and the  $\gamma$ -band at 450  $m\mu$ .

This interpretation would also eliminate the following objection which has been raised (9). In discussing the intensities of the absorption bands of the cytochrome  $a$  and  $a_3$  components, Keilin and Hartree state that the  $\alpha$ -band of cytochrome  $a_3$  is weak compared with that of cytochrome  $a$ , whereas this relationship is reversed in the case of the  $\gamma$ -bands. This relationship places a weak  $\gamma$ -band together with a strong  $\alpha$ -band, a situation which has not yet been found to exist in the case of iron-porphyrins. If the bands found by the photochemical method are assigned to the CO compound of cytochrome  $a_3$ , this objection would be avoided; for then the weak band at 452  $m\mu$  would be the  $\gamma$ -band of the porphyrin possessing the weak  $\alpha$ -band at 590  $m\mu$ . This, however, implies that the strong  $\gamma$ -band of cytochrome  $a$  at 450  $m\mu$  shifts in the presence of CO to 432  $m\mu$ .

In this connection it is of interest to note that Keilin and Hartree place the  $\alpha$ - and  $\gamma$ -bands of the cyanide complex of cytochrome  $a_3$  at 590 and at 450  $m\mu$ , which are the positions also found by the photochemical experi-



ments for the CO compound of cytochrome oxidase. One should also keep in mind the apparent shift of the  $\gamma$ -band, but not of the  $\alpha$ -band, of cytochrome *a* when KCN is added to the reduced enzyme preparation in the presence of air.

*Relationship of Cytochrome Oxidase to Pasteur Enzyme*—The Pasteur enzyme of rat retina has been found photochemically also to have its main absorption band at 450 m $\mu$ ; however, its non-identity with cytochrome oxidase of rat heart muscle is indicated by the fact that the  $\alpha$ -bands are located at different positions; namely, at 578 m $\mu$  for the Pasteur enzyme, and at 589 m $\mu$  for cytochrome oxidase. A similar situation exists in the yeast cell, in which the  $\gamma$ -bands of the Pasteur enzyme and the respiratory ferment coincide, whereas the structure of the  $\alpha$ -bands differ significantly.

#### SUMMARY

The absorption spectrum of the CO compound of cytochrome oxidase from rat heart muscle has been charted by Warburg's photochemical method. The main absorption band is located near 450 m $\mu$ , and two secondary maxima are situated at 510 and 589 m $\mu$ , indicating that cytochrome oxidase belongs to the class of pheohemin enzymes.

The question of the identity of cytochrome oxidase and the cytochrome  $a_3$  component of Keilin and Hartree has been discussed in the light of the present findings.

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# A SUGGESTED MECHANISM OF BIOLOGICAL ACYLATIONS

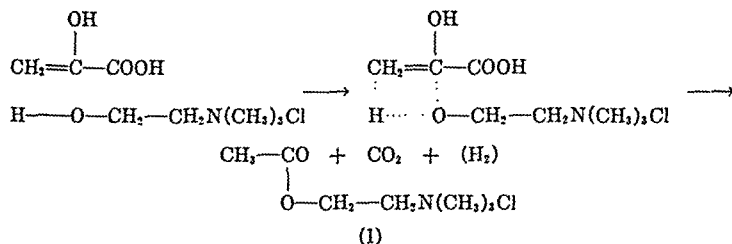
## I. THE FORMATION OF ACETYLCHOLINE

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Our knowledge of the biochemical reactions which lead to the formation of acetylcholine *in vivo* is still inadequate. Previous investigations (1, 2) indicate that choline (or some derivative of choline) reacts with an intermediate compound of carbohydrate metabolism to form a complex which, on enzymatic dehydrogenation, yields acetylcholine.<sup>1</sup> Two metabolites, namely pyruvic acid and acetoacetic acid, appear to be theoretically capable of participating in the synthesis of acetylcholine. Stedman and Stedman (1), assuming that pyruvic acid is the metabolite, proposed the reaction scheme (I).



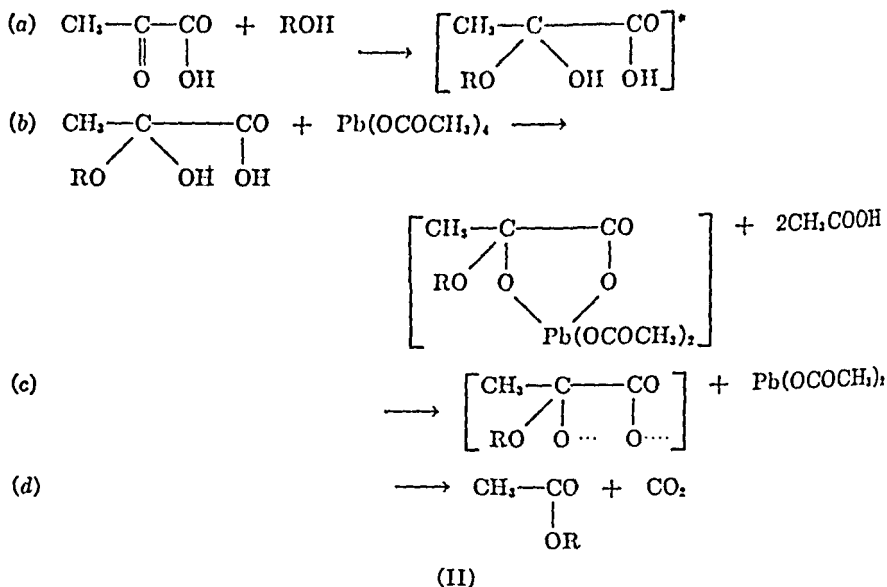
This scheme seems to express reasonably well the known biochemical facts concerning the synthesis of acetylcholine *in vivo*. It is open, however, to one objection. The known additive compounds of pyruvic acid, *e.g.* those which are obtained by the addition of hydrogen sulfide (4), mercaptan (5), or thioglycolic acid (6) and which are stable enough for isolation, belong to the keto rather than the enol form of this acid. More recently (7) the first type of addition was also found to occur in a number of other  $\alpha$ -keto acid adducts. The formulation of the hypothetical additive compound in Stedman's scheme as a derivative of the enol pyruvic acid seems therefore questionable.

Experience gained by the author in recent investigations concerning the oxidative cleavage of  $\alpha$ -keto acids by means of lead tetraacetate permits

<sup>1</sup> According to Lipmann (3) there exists the further possibility that the biological formation of acetylcholine may be caused by the action of acetyl phosphate on choline.

the suggestion of a modified reaction scheme for the formation *in vivo* of acetylcholine, which seems to correspond more accurately with the chemical facts presented below.

It has been demonstrated (7) that if the cleavage of  $\alpha$ -keto acids by means of lead tetraacetate (LTA) is carried out in the presence of alcohols esters are obtained. The resulting esters contain acids with 1 carbon atom less than the original keto acids. The reaction has been carried out with a variety of  $\alpha$ -keto acids (and  $\alpha$ -keto alcohols) in the presence of methanol, ethanol, and benzyl alcohol and has in each instance produced good yields of the corresponding esters.<sup>2</sup> The chemical reaction involved has been elucidated. The supporting evidence has been set forth *in extenso* in the first two papers (7) on this subject and will not be dealt with here. The course of the reaction is illustrated in the scheme (II, a-d) for pyruvic acid, since the oxidative cleavage of this acid is of particular interest in connection with the present investigation.



\* The brackets indicate that the compound has not yet been isolated.

Two observations made during the investigations must be stressed, as they influence the decision to be reached concerning the course of the chemical as well as the biochemical acylation reaction: it was found that

<sup>2</sup> It is obvious that the oxidative cleavage reaction, if carried out in the presence of alcohols, may be regarded as an esterification process.

the initial formation of a loose additive compound<sup>3</sup> between the  $\alpha$ -keto acid and the alcohol is indispensable for the reaction and that in forming the adduct the  $\alpha$ -keto acid reacts only in its keto form. The constitution of these adducts is established with a reasonable degree of certainty, as illustrated in scheme (II, a).

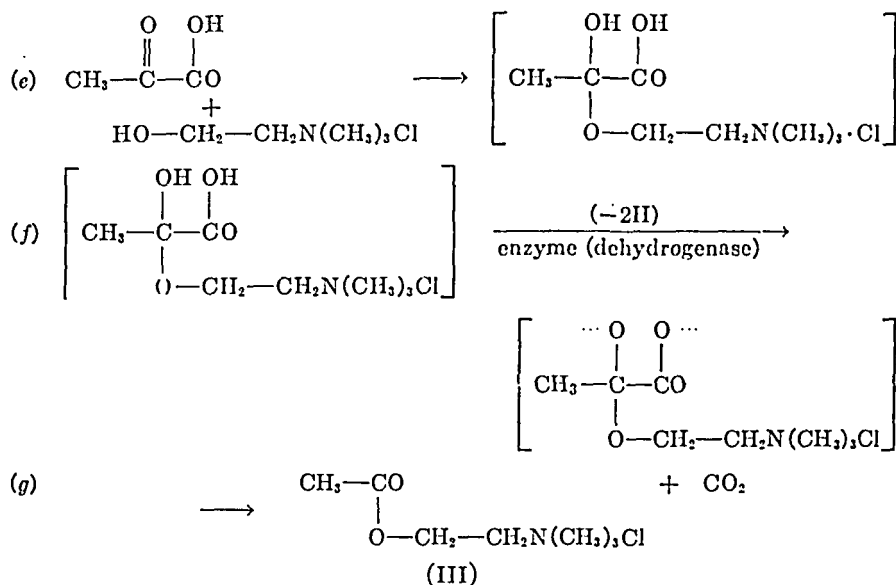
Having succeeded, in a number of cases, in obtaining esters of alcohols by subjecting simple alcohols to the *oxidative acylation*, it seemed worth while to determine whether this reaction, if applied to choline, would form choline esters, in particular acetylcholine. Such has been found to be the case. The reaction proceeded smoothly, giving good yields of the acylation products of choline. The reaction scheme (a-d) will illustrate the acetylation of choline if  $-\text{CH}_2-\text{CH}_2\text{N}(\text{CH}_3)_3\cdot\text{Cl}$  is substituted for R.

The chemical acylation of choline was easily realized by experiment. Choline chloride and choline bromide are commercially available and are a convenient source of pure choline. Both salts, however, react with lead tetraacetate, forming the insoluble lead halides. They were therefore transformed into the acetate with silver acetate. After preliminary tests, glacial acetic acid proved to be a convenient solvent in which to perform this transformation as well as the acylation reaction itself. In order to obtain good yields of the acylation products all reagents and the solvent must be thoroughly dry. The reaction, which was carried out by adding LTA to an equimolecular mixture of choline and the  $\alpha$ -keto acid (e.g., pyruvic acid or phenylglyoxylic acid), took place immediately with a rise in temperature. The choline esters were isolated in the form of their chloroaurates and chloroplatinates. The rather soluble acetylcholine chloroaurate was obtained with a yield of 76 per cent; the only very slightly soluble chloroaurate and chloroplatinate of benzoylcholine, however, were obtained with nearly theoretical yields, thus indicating the quantitative course of the reaction. In the case of acetylcholine care was taken to ascertain that its formation was due only to the new oxidative acylation reaction and not to a possible acetylating action of the solvent or lead tetraacetate. This was established by repeating the procedure with either the keto acid or lead tetraacetate omitted. In both cases only unchanged choline chloroaurate could be obtained as the final product. Further evidence along these lines was the almost quantitative conversion of choline to benzoylcholine in glacial acetic acid.

As previously pointed out, it has been assumed that the biosynthesis of acetylcholine is the result of the enzymatic dehydrogenation of a loose additive compound between pyruvic acid and choline. The newly discovered chemical acylation of choline is so strikingly similar in many

<sup>3</sup>The additive compound may be associations of molecules held together weakly by physical rather than by chemical forces.

respects to the biosynthesis of acetylcholine that by comparing the two processes it has become possible to propose a modified reaction mechanism, scheme (III), for its formation *in vivo*. The reaction scheme remains essentially that of Stedman and Stedman but contains the alteration necessitated by the present investigation.



Stedman and Stedman's concept of the initial formation of a pyruvic acid-choline adduct is retained, the only change suggested being that the addition of choline takes place at the non-enolized keto group. The adduct serves as substrate for the *enzymatic* dehydrogenation. The resulting unstable radical by spontaneous disintegration, involving the liberation of carbon dioxide, yields acetylcholine. Apart from the change mentioned, Stedman and Stedman's formulation of the biochemical reaction leading to the synthesis of acetylcholine is corroborated by the present investigation.

As a mere speculation it is suggested that the action of an appropriate enzyme upon the adduct leads to the transitory formation and decomposition of intermediates similar in constitution to those postulated for the chemical acylation (*cf.* (II, *a-d*) and also (7)).

The observations made in the course of the present investigation and recent publications of other investigators warrant the expectation that the oxidative cleavage of the adducts of  $\alpha$ -keto acids with alcohols may be a fundamental reaction in bringing about biological acylations. Thus according to Lipmann (3) acetyl phosphate, a recently discovered intermediate, is also believed to be formed *in vivo* by an oxidative acylation.

Chemical evidence supporting Lipmann's formulation of the biosynthesis of acetyl phosphate will be given in a subsequent paper.

There is also chemical evidence that oxidative acylation may play a rôle in the synthesis of glycerides *in vivo*. Investigations concerning the oxidation of fatty acids by Witzemann (8) and Dakin lead one to expect that the  $\alpha$ -keto acids needed for such an acylation may be formed *in vivo*. If in the reaction (*cf.* (II, *a-d*)) the pyruvic acid is replaced by a higher member of the series of  $\alpha$ -keto fatty acids and the alcohol is glycerol (or a derivative thereof), the reaction product would be a glyceride. Chemical evidence supporting the concept of such a biological synthesis of glycerides by oxidative acylation has been obtained.

#### EXPERIMENTAL

*Acetylcholine*—A solution of choline acetate in glacial acetic acid was found to be suitable for demonstrating the acylation of choline by means of  $\alpha$ -keto acids and LTA.

*Preparation of Choline Acetate*<sup>4</sup>—A mixture of 0.7 gm. of dry choline chloride and 0.85 gm. of dry silver acetate in 10 cc. of dry acetic acid was kept at 50° for a period of 5 minutes with occasional shaking. The resulting solution (A) of choline acetate in glacial acetic acid was used without removal of silver chloride.

*Acetylation*—To a cooled mixture of Solution A and 1 gm. of carefully purified pyruvic acid were added 2.5 gm. of finely powdered LTA in small portions over a period of 5 minutes. The temperature of the mixture was kept from rising above 20° by cooling with water. The reaction took place immediately with the evolution of carbon dioxide and the formation of acetylcholine.

*Isolation of Acetylcholine As Chloroaurate*—The solution of acetylcholine in acetic acid was freed of silver chloride by centrifuging and the precipitate was washed twice with glacial acetic acid. The combined solutions were concentrated *in vacuo* (10 mm., bath at 25°) to a thick syrup. In order to separate the lead ion, the residue was taken up with 25 cc. of ice-cold water and mixed with a saturated aqueous solution of 0.85 gm. of sodium sulfate and filtered. Traces of silver, if any were present, were removed by adding 2 N HCl dropwise to the cooled filtrate as long as a precipitate continued to form. The silver chloride was filtered off. Upon further addition of 0.5 cc. of 2 N HCl and a solution of 2.6 gm. of auric chloride in 6.0 cc. of water, the acetylcholine chloroaurate precipitated immediately.

<sup>4</sup> All reagents used, especially pyruvic acid, were of a high grade of purity. Since the affinity of pyruvic acid is greater for water than for choline, the formation of the necessary pyruvic acid-choline adduct will be partly or completely prevented, according to the amount of water present. The reactions therefore must be carried out with dry reagents and with suitable precautions against the intrusion of moisture.

To increase the yield the mixture was allowed to stand 1 hour on ice. The bright yellow salt was thoroughly freed from its mother liquor by filtration with suction, washed with a few cc. of ice-cold water, and dried *in vacuo*, giving 1.87 gm. (76 per cent) of crude acetylcholine chloroaurate, m.p. 153–160°. After two recrystallizations (from 8 cc. portions of boiling water) 0.66 gm. of pure compound, m.p. 162–164°, was obtained. A mixed melting point with an authentic sample showed no depression.

$C_7H_{16}O_2NCl \cdot AuCl_3$  (485.2)

Calculated. C 17.35, H 3.29, Au 40.6, acetyl\* 8.86

Found. " 17.32, " 3.26, " 40.65, " 9.36

\* The chloroaurate was decomposed with metallic silver (9) and the acetyl titrated with 0.01 N sodium hydroxide (10).

*Isolation of Acetylcholine Chloride*—The gold salt was dissolved in water and decomposed with metallic silver, according to the procedure of Dudley (9).

$C_7H_{16}O_2NCl$  (181.5). Calculated, C 46.3, H 8.8; found, C 46.8, H 9.0

*Benzoylcholine*—Owing to the nature of phenylglyoxylic acid, the procedure had to be slightly modified.

The solution of choline acetate was prepared as described above, with the exception that 20 cc. of glacial acetic acid were used as solvent. To this were added 1.5 gm. of phenylglyoxylic acid and 2.5 gm. of LTA. The mixture was shaken for 1 hour with occasional warming to 40° and kept at room temperature for a further 2 hours. The benzoylcholine was isolated as chloroaurate by the procedure given above. In order to obtain the analytically pure gold salt of benzoylcholine it was sufficient to wash the crude precipitate with water until the filtrate was colorless and to dry the residue *in vacuo*. The yield of benzoylcholine chloroaurate was almost quantitative, 2.70 gm., m.p. 187–188°. Fourneau and Page (11) reported 182°.

$C_{12}H_{18}O_2NCl \cdot AuCl_3$  (547.1)

Calculated. C 26.3, H 3.32, Au 36.0, benzoyl\* 22.11

Found. " 26.3, " 3.45, " 36.15, " 21.50

\* Determined as benzoic acid, which was isolated in substance; m.p. 122–123°.

*Isolation of Benzoylcholine from Its Gold Salt*—561 mg. of benzoylcholine chloroaurate, suspended in 50 cc. of 95 per cent ethanol kept at 60°, were vigorously stirred with 2 gm. of finely divided metallic silver until the solution became colorless (2 minutes). The filtrate was concentrated *in vacuo* (10 mm., bath at 25°) and the residue brought to dryness in a

vacuum desiccator ( $\text{CaCl}_2$ ). Yield of benzoylcholine, 240 mg. (96 per cent). For purification, the substance was redissolved in 2 cc. of ethanol, centrifuged, and brought again to dryness. M.p. 204–205°; Fournneau and Page (11), 200°.

$\text{C}_{12}\text{H}_{18}\text{O}_2\text{NCl}$  (243.6). Calculated, C 59.11, H 7.44; found, C 59.23, H 7.06

*Benzoylcholine Chloroplatinate*—The procedure was repeated and the benzoylcholine was precipitated as the chloroplatinate. The substance was washed with water until the filtrate was colorless and then dried *in vacuo*. The salt thus obtained was analytically pure. M.p. 234°, with decomposition; Fournneau and Page (11), 224°. The yield was nearly quantitative (99.1 per cent).

$(\text{C}_{12}\text{H}_{18}\text{O}_2\text{NCl})_2 \cdot \text{PtCl}_4$  (824.2)

Calculated, C 34.95, H 4.38, Pt 23.7; found, C 35.14, H 4.02, Pt 23.8

#### SUMMARY

The acetylation of choline by means of pyruvic acid and lead tetraacetate is described. This oxidative acylation offers a plausible explanation for the chemical reaction involved in the biological formation of acetylcholine.

It is suggested that enzymatic *oxidative acylation* may be fundamental in bringing about biological acylations. The probable course of the reaction has been proposed.

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# A SIMPLE METHOD FOR THE CHEMICAL DETERMINATION OF URINARY THIAMINE BASED UPON THE PREBLUDA-McCOLLUM REACTION\*

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The methods now available for the estimation of urinary thiamine may be classified as biological (1-4) or chemical. The former are open to many objections, among which are lack of specificity, accuracy, and practicability for routine laboratory use.

The chemical methods are based chiefly upon two reactions. In one the observation of Barger *et al.* (5) that thiamine can be oxidized to a yellow-blue fluorescent compound, later identified as thiochrome, is employed. Jansen (6) applied this observation to the assay of thiamine in various biological materials, and since then numerous workers (7-10) have, by various modifications, devised techniques which have been used in the study of thiamine nutrition. Although the thiochrome method is extremely sensitive, it has been found to lack specificity (11-14)<sup>1</sup> because of the presence in normal urine, and in the urine of subjects taking certain drugs, of variable amounts of thiochrome and other fluorescent compounds for which due correction must be made. The conversion of thiamine to thiochrome is, furthermore, only approximately two-thirds complete (15). A conversion factor is therefore required for all determinations. There are other difficulties inherent in the method. The occurrence of certain substances in abnormal urine (15), for example, interferes with the quantitative conversion of thiamine to thiochrome and, by their own fluorescence, with the thiamine determination itself (6, 8, 9, 16, 17). It is difficult to correct not only for such factors (5, 17), but also for other substances which may affect the intensity and shade of the fluorescence caused by thiochrome (15, 18, 19). In addition, the need for an ultraviolet fluorometer and a skilled technician well experienced in its use precludes the use of this method from most routine clinical laboratories.

There are several colorimetric chemical methods for measuring thiamine, all of which are based upon the Ehrlich-Pauly reaction in which the vitamin is coupled with a diazotized amino compound. Prebluda and McCollum (20) have described a dye which was produced by the coupling of thiamine

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<sup>1</sup> Holt, L. E., and Najjar, V. A., personal communication.

with diazotized *p*-aminoacetophenone. Melnick and Field (21) applied this reaction to the measurement of urinary thiamine. Although their method is highly specific, it is too complex, laborious, and time-consuming for clinical use, and in the hands of other workers it has presented insurmountable difficulties. Although this method involves a simple chemical reaction, its complexity lies in the removal of substances which interfere both with the selective adsorption of the vitamin on permutit and with the coupling reaction.

Recently Kirsh and Bergeim (22) described a method for the determination of urinary thiamine in which the vitamin is coupled with diazotized ethyl *p*-aminobenzoate. Although this technique avoids the use of a selective adsorbent, its sensitivity is limited to more than 3.0  $\gamma$  of thiamine per 5.0 cc. of urine. Consequently, concentration of the urine is necessary for measurements on 24 hour urine samples. The method is not applicable to the study of fractional excretion in which small amounts of thiamine must be assayed. Furthermore, adrenalin and other compounds produce colored substances with this reagent which disappear only on standing 8 to 12 hours.

Emmett, Peacock, and Brown (23) have demonstrated that thiamine can be quantitatively adsorbed by superfiltrol<sup>2</sup> from water and certain biological solutions and that the Prebluda-McCollum reaction will take place with the thiamine thus adsorbed. The resulting dye can then be quantitatively eluted from the adsorbent and the intensity of the color compared with that of a standard solution treated similarly. The Emmett, Peacock, and Brown technique satisfies the requirements for a specific, accurate, and practical method for measuring thiamine. The presence in urine of interfering substances, however, prevents its application to the measurement of the vitamin in this medium. We have identified two of these interfering substances. By applying a few simple procedures, we have developed a method whereby these can be eliminated and urinary thiamine measured by the Emmett, Peacock, and Brown technique.

#### EXPERIMENTAL

*Identification of Interfering Substances*—Attempts to identify the substances in urine which interfere with the selective adsorption of thiamine and its reaction with diazotized *p*-aminoacetophenone were made by experiments on a solution made up to contain thiamine and other constituents of urine in their physiological concentrations. 1 liter of this solution contained the following substances: thiamine chloride hydrochloride 0.5 mg., sodium chloride 9.0 gm., sodium monohydrogen phosphate 1.0 gm., ammonium chloride 0.5 gm., sodium sulfate 1.0 gm., calcium chloride 0.5 gm.,

<sup>2</sup> An adsorbing agent manufactured by the Filtrol Corporation, Los Angeles.

urea 20.0 gm., creatinine 1.0 gm. Recovery of thiamine from this solution by adsorption with superfiltrol was complete. Of these constituents, only ammonium chloride was found to decrease the yield. This occurred only when the concentration of this salt was much greater than that in normal or abnormal urine.

When uric acid was added in amounts of 0.6 gm. per liter, there was a loss of approximately 50 per cent in the recovery of thiamine. From the color of the coupling reaction it appeared that uric acid interfered with this step.

The presence of ascorbic acid also resulted in large losses of thiamine. Other observers (22, 23) have found that this substance interferes with the coupling reaction of thiamine. Kirch and Bergeim (22) circumvented this by oxidation of the ascorbic acid with potassium permanganate solution. While Emmett, Peacock, and Brown (23) avoided the effect of small amounts of ascorbic acid by adsorbing the thiamine on superfiltrol, this

TABLE I  
*Effect of Ascorbic Acid on Thiamine Recovery from Saline Solution*

Ascorbic acid concentration	Thiamine recovery
<i>mg. per cent</i>	<i>per cent</i>
0	100
0.5	100
1.0	84
2.0	73
3.0	66
5.0	60

method failed in the presence of concentrations comparable to those found in urine. The loss due to ascorbic acid was related to its concentration and, in the presence of amounts found in the urine of well nourished individuals, was as high as 40 per cent (Table I).

*Removal of Interfering Substances*—It was found that the effect of uric acid on the thiamine determination could be eliminated by precipitation of the uric acid with zinc acetate and sodium carbonate solutions, according to the method of Morris (24) who used this technique for the measurement of uric acid in urine. In our experiments there was no loss of thiamine as a result of this precipitation.

More difficulty was encountered in attempts to avoid the interference from ascorbic acid. Although we found that thiamine could be quantitatively adsorbed from a simple solution containing ascorbic acid at pH 7 instead of the usual pH 4.5, this procedure was unsuccessful when applied to urine. Attempts to oxidize the ascorbic acid by 2,6-dichlorophenol

indophenol and dilute solutions of iodine were also unsatisfactory. In our search for a precipitating agent for ascorbic acid, we found that in 1925 Zilva (25) had used basic lead acetate to precipitate the antiscorbutic factor from lemon juice. Other observers (26-28) have found also that lead acetate at alkaline pH would precipitate many impurities in biological solutions containing thiamine without appreciable loss of this vitamin. On the basis of these observations, basic lead acetate precipitation was employed to eliminate ascorbic acid and other interfering substances from urine.

*Method of Thiamine Determination on Urine*—Identification of interfering substances and their elimination by the procedures discussed above permitted the development of a simple method for the determination of urinary thiamine. The technique was as follows:

The urine was collected in the presence of toluene and enough 10 per cent sulfuric acid to maintain a pH of 3 or less (about 20 cc. of 10 per cent sulfuric acid are necessary for a 24 hour urine). It was found advisable to let the urine stand 24 hours or longer before analysis, particularly in the case of concentrated urines. This favored urate precipitation. Under such conditions, the thiamine content remained constant for at least 1 month.

To 100 cc. of filtered urine in a 250 cc. centrifuge flask were added 5.0 cc. of 20 per cent zinc acetate solution. A precipitate of zinc phosphate which occasionally formed was redissolved by the addition of a few drops of 10 per cent hydrochloric acid. The solution was alkalinized to approximately pH 7.4 by the slow addition of saturated sodium carbonate solution with constant agitation. 5 cc. of the zinc acetate solution were again added.

10 cc. of lead acetate reagent<sup>3</sup> were added slowly with constant agitation. The pH, which fell during this addition, was then brought to approximately 8.2 by the slow addition of saturated sodium carbonate solution. The mixture was then centrifuged and filtered into another centrifuge flask containing 12 cc. of 10 per cent hydrochloric acid. The precipitate was washed twice with about 15 cc. of water to which were added a few drops of the saturated sodium carbonate solution.

The filtrate and washings were pooled and adjusted to pH 4.5 and were then adsorbed with 600 mg. of superfiltrol for 1 hour, as outlined by Emmet, Peacock, and Brown (23). The mixture was centrifuged; the supernatant was decanted into another centrifuge flask and reabsorbed with another 600 mg. of superfiltrol for 1 hour, at which time it was centrifuged and the supernatant discarded. In this way a small fraction of the thiamine which escaped adsorption was recovered. The two adsorbates were then treated separately.

<sup>3</sup> 100 gm. of neutral lead acetate were dissolved in 200 cc. of distilled water; 2 N sodium hydroxide was added to pH 7.5.

The adsorbed thiamine was coupled with the alkalinized diazotized *p*-aminoacetophenone reagent, with slight modification of the method described by Emmett, Peacock, and Brown. To each adsorbate were added 5.0 cc. of water, 5.0 cc. of 95 per cent alcohol which contained 5.0 mg.

TABLE II  
*Analysis of Urinary Thiamine and Recovery of Added Thiamine*

Subject	Thiamine per 100 cc. urine	Thiamine per 100 cc. urine + 50 $\gamma$	Added thiamine recovered		24 hr. output	Remarks
	$\gamma$	$\gamma$	$\gamma$	per cent	$\gamma$	
N. Z.	17.8	61.0	43.2	86.4	167	Normal subject
E. S.	16.0	58.9	42.9	85.8	162	" "
M. B.	13.3	57.8	44.5	89.0	206	" "
B. A.	9.9	52.2	42.3	84.6	105	" "
E. L.	29.8	72.5	42.7	85.4	471	" "
	29.4	70.9	41.5	83.0	478	
M. L.	9.3	51.3	42.0	84.0	136	" "
	10.4	51.4	41.0	82.0	156	
A. L.	12.9	56.2	43.3	86.6	103	" "
S. L.	14.8	55.9	41.1	82.2	144	" "
	14.3	56.3	42.0	84.0	136	
H. H.	15.4	54.7	39.3	78.6	202	" "
	14.5	57.9	43.4	86.5	135	
I. R.	7.4	49.6	42.2	84.4	112	" "
F. G.	5.4	49.7	44.3	88.6	84	" poor dietary habits, low intake
R. G.	7.4	52.9	45.5	90.0	59	Normal; unsatisfactory reducing diet
B. A.	6.4	50.6	44.2	88.4	72	Diarrhea; gastrointestinal upset
G. F.	129.1	171.9	42.8	85.6	1423	Rheumatic heart disease; congestive failure; myxedema; thiamine 3 mg. daily
P. K.	26.4	67.0	40.6	81.2	332	Diabetes; hypertension; cardiovascular disease; thyrotoxicosis; (taking brewers' yeast)
F. S.	0.4	45.4	45.0	90.0	4.4	Polycythemia vera; vascular nephritis; nitrogen retention; diabetes mellitus
	0.1	43.6	43.5	87.0		
C. D.	5.7	49.6	43.9	87.8		Obstructive jaundice due to gallstones
Average.....				86 (A.D. $\pm$ 2%)		

of phenol per cc., and a few drops of thymol blue (alkaline). The mixture was adjusted to pH 7 to 8 with 0.5 N sodium hydroxide and 10 cc. of the alkalinized diazotized reagent were added immediately with thorough mixing. The mixture was allowed to stand for 2 hours or longer and was

then filtered by suction on a small Hirsch filter. The precipitate and flask were washed three times with a total of 5.0 cc. of water.

The adsorbate and filter paper were transferred to a dry centrifuge tube. 4 cc. of 95 per cent alcohol were run into the centrifuge tube through the Hirsch filter. The tube was then tightly stoppered, shaken vigorously, and centrifuged. (If amounts of thiamine greater than 50  $\gamma$  were present, 8.0 cc. of 95 per cent alcohol were used.)

The intensity of the color in the alcoholic eluate was measured in the Evelyn photoelectric colorimeter which had been previously calibrated with known amounts of thiamine handled similarly. The 540  $m\mu$  filter was used.

### Results

By the technique described above, twenty-two determinations were made on nineteen urine samples from sixteen individuals, comprising both nor-

TABLE III  
*Duplicability of Thiamine Analyses on Urine*

Sample No . . . . .	1	2	3	4	5	6	7	8
Thiamine per 100 cc. urine, $\gamma$	29.8 29.4	9.3 10.4	14.8 14.3	135.4 134.2 129.1	72.5 70.9	51.3 51.4	55.9 56.3	175.4 174.8
Difference, %	1.4	11.2	3.4	4.8	2.2	0.2	0.7	0.3

A.D.,  $\pm$  3 per cent.

mal and abnormal subjects. The abnormal groups consisted of patients with diabetes mellitus, chronic nephritis, polycythemia vera, myxedema, hyperthyroidism, dietary deficiency, and obstructive jaundice. Determinations were made on two aliquots of the same 24 hour urine specimen, to one of which were added 50  $\gamma$  of thiamine.

The results of these analyses are shown in Table II. The average recovery of added thiamine was 86.0 per cent (A.D. =  $\pm$ 2). Twenty-one of these recovery determinations fell between 80 and 90 per cent. In eight experiments in which duplicate determinations were made on the same sample (Table III) the average discrepancy between duplicates was 3.0 per cent. All of these but one checked to within 5.0 per cent.

### DISCUSSION

The identity of the chief substances in urine which interfere with the Prebluda-McCollum reaction of thiamine has been established. Although the mechanism by which these substances interfere is not clear, their pre-

precipitation without appreciable loss of thiamine has permitted the development of a simple method by which this vitamin may be measured in human urine. The specificity of the chemical reaction upon which this method is based has been amply demonstrated by other workers (20, 21, 23), and our experience with this reaction has been in complete accord with these findings.

The method herein described permits the determination of urinary thiamine by a technique so simple as to make it readily available to all routine clinical laboratories. A single analysis requires 5 hours, 4 of which are spent in permitting reactions to take place. Six or more analyses may be conveniently undertaken simultaneously with but little increase in the time required. The amount of urine necessary for one determination furthermore, is so small, in contrast to that required by other specific methods, as to permit studies of the fractional excretion of thiamine.

Although objections may be raised to the fact that the recovery of added thiamine is not complete, our recovery of 86 per cent compares favorably with that of other methods, and its simplicity warrants its use when a 14 per cent loss is of little significance. For more accurate analyses a simultaneous recovery determination may be carried out in order to establish accurately the percentage loss by means of which due correction may be made.

It has been found that if less urine is taken for analysis, better recovery of added thiamine ensues. The lowest yields were obtained, furthermore, on very concentrated urines. For these reasons it is considered desirable to dilute all 24 hour urine outputs of less than 1000 cc. to 1 liter, an aliquot of which may be taken for analysis.

The 24 hour urinary excretion of thiamine in eleven normal subjects ranged between 103 and 478  $\gamma$ . In two subjects who appeared normal but whose dietary habits were irregular, the total daily thiamine output was 84 and 59  $\gamma$  respectively. The latter subject was on a reducing diet, the thiamine content of which was 0.633 mg. (calculated from accepted standards of food values).

The lowest 24 hour thiamine excretion obtained in our series occurred in a patient with chronic vascular nephritis and nitrogen retention. It is difficult to explain the almost negligible excretion of thiamine in this case on the basis of deficiency; more probably, this is a reflection of impaired renal function.

#### SUMMARY

1. The chief substances in urine which interfere with the Prebluda-McCollum reaction for thiamine have been identified.
2. Removal of these substances by precipitation has permitted the



application of this specific chemical reaction to the measurement of urinary thiamine.

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# THE HEMICELLULOSES OF FORAGE PLANTS\*

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The chemistry of the cell wall constituents of pasture grasses with some exceptions has been dealt with in terms of crude fiber and pentosans. The first term fails to include the subject of the discussion (1); the second yields information which we are not able to apply accurately. Because pasture grasses play an important rôle in our economic system and because in some instances the polyoses and polyuronide hemicelluloses are present to a considerable extent, there seems to be some justification for investigations on these substances. Even with concrete evidence of the identity of the constituents of these bodies, there still remains the problem of ascertaining their place in the metabolism of the plant. In other words, the chemistry of the hemicelluloses remains to be written (2).

This report deals with an analysis of the total polyuronide hemicelluloses of two species of grass, sheep's fescue (*Festuca ovina*) and sweet vernal grass (*Anthoxanthum odoratum*). In addition to the generally known differences in structure of these two grasses there are at least two variations in chemical composition which should be pointed out at this time. Under our cultural conditions sheep's fescue is characterized by a high content of crude fiber and a low content of moisture, which is in direct contrast to the composition of sweet vernal grass.

## EXPERIMENTAL

The material representing each species was a composite of samples which had been collected throughout the growing season for several years. The grass plots had been kept in a vegetative condition and were substantially free from weeds.

The following methods of analysis were used on finely ground material: Total ash and total furfural were determined by the methods of the Association of Official Agricultural Chemists (3); total hemicelluloses, by the Buston method (4); arabinose, by the Wise and Peterson modification (5) of the Neuberg and Wohlgemuth method (6); uronic acid anhydrides, by

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the Phillips, Goss, and Browne modification (7) of the Dickson, Otterson, and Link method (8). The polyuronide hemicelluloses were isolated and hydrolyzed by a procedure similar to that used by Weihe and Phillips (9); the lignin was removed by sodium hypochlorite.

On a quantitative basis sheep's fescue yielded 18.11 per cent of polyuronide hemicelluloses; sweet vernal grass yielded 12.05 per cent. The syrup from the hydrolyzed material yielded osazones characteristic of arabinose and xylose. The specific rotations of the syrups were  $+28^\circ$  and  $+41.6^\circ$  at  $20^\circ$ , respectively. These values correspond quite well with those one should obtain from such a mixture of sugars when present in the proportions found later.

On an ash- and moisture-free basis the isolated polyuronide hemicelluloses from sheep's fescue had the following composition in per cent: total furfural 50.97, uronic acid anhydride 6.15, furfural from uronic acid anhydrides 1.18, *l*-arabinose estimated to be not more than 1 per cent by the diphenylhydrazine test, furfural from *l*-arabinose 0.59, *d*-xylose 82.02, furfural from *d*-xylose (by difference) 49.20, glucose present as indicated by the saccharic acid test, galactose not detected, remainder 10.83.

On the same basis the polyuronide hemicelluloses from sweet vernal grass had the following composition in per cent: total furfural 45.46, uronic acid anhydrides 7.69, furfural from uronic acid anhydrides 1.47, *l*-arabinose 18.87, furfural from *l*-arabinose 9.06, *d*-xylose 60.66, furfural from *d*-xylose (by difference) 34.93, glucose present as indicated by the saccharic acid test, galactose not detected, the remainder in this case 12.78.

The foregoing data indicate that the molar ratio of the uronic acid anhydride to *l*-arabinose and *d*-xylose in the polyuronide hemicellulose of sheep's fescue is 1:0.2:15.7; of sweet vernal grass, 1:2.9:9.3.

#### DISCUSSION

In general, it has been observed that the hemicelluloses of lignified tissue are of the glucosan-xylan type; in the non-lignified tissues they are of the galacto-araban type (10).

In this work, dealing entirely with lignified tissue, both types are present but the former class of hemicelluloses is displaced to a greater extent by the latter in the species having the greater content of water. In the grass with the lower content of water the above generalization holds true.

A preliminary examination of the viscosity of the respective polyuronide hemicelluloses indicated that those from sweet vernal grass when dispersed in water produced not only the more viscous system but also the more stable system; those from the sheep's fescue for the same period and under the same conditions were almost completely flocculated. These observations would indicate that the former has the greater degree of hydration.

It is realized that the viscosity of a system may be altered by several factors. However, attempts were made to eliminate all variables except those differences inherent in the dispersed phase. Alterations purposely applied did not alter the gross results. It seems therefore that the hemicelluloses of sweet vernal grass must contribute considerably to its high moisture content. If the observed behavior of the hemicelluloses of sheep's fescue is responsible for its low moisture content, the difference noted in the structure of the two hemicelluloses assumes an important rôle. For instance, of what significance is *l*-arabinose to the process of hydration?

#### SUMMARY

The polyuronide hemicelluloses of sheep's fescue (*Festuca ovina*) and sweet vernal grass (*Anthoxanthum odoratum*) in the vegetative state were isolated. These products when hydrolyzed yielded a uronic acid, *l*-arabinose, and *d*-xylose in the approximate molar ratio of 1:0.2:15.7 and 1:2.9:9.3 respectively. The degree of hydration of the two products appeared to differ considerably. The species containing the more highly hydrated product had the greater original moisture content and contained the larger percentage of *l*-arabinose.

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# THE INACTIVATION OF AMINE OXIDASE BY ENZYMATIC OXIDATIVE PRODUCTS OF CATECHOL AND ADRENALIN\*

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Adrenalin, by virtue of its particular chemical configuration, is subject to two different kinds of enzymatic oxidation. Like many other hydroquinones, it can be oxidized by the cytochrome oxidase system and by polyphenoloxidase (1, 2). The end-product for this quinone oxidation of adrenalin is a group of red compounds called adrenochrome, capable of condensing into melanin-like pigments. On the other hand, the side chain of the adrenalin molecule has the structure of an aliphatic amine, and as such may undergo oxidative deamination through the action of amine oxidase (3).

The studies of Gaddum and Kwiatkowski (4) and many others have shown that the attack on adrenalin by the amine oxidase is destructive of the pharmacological activity of adrenalin, at least on those organs in which the action of adrenalin is exciting, and that the inhibition of the amine oxidase by ephedrine and related drugs accounts, in part, for the sympathomimetic action of these drugs. On the other hand, Friedenwald and Buschke<sup>1</sup> have shown that a cyanide-sensitive system is necessary to activate the adrenergic reaction and that under certain conditions cytochrome *c* may be shown to act as a link in this activating system. It does not necessarily follow that the quinone oxidation of adrenalin is a step in the adrenergic reaction. Still it was of interest to discover how these two oxidizing systems which have opposing effects on many adrenergic reactions may compete in the tissue for adrenalin as substrate.

In tissues in which both the quinone-oxidizing and the amine-oxidizing systems are active, either one can be separately suppressed, the former by the addition of cyanide, the latter by benzedrine. On the other hand, tissue preparations are readily obtained which contain abundant cytochrome oxidase and amine oxidase but which are relatively deficient in cytochrome *c*. Such preparations are unable to oxidize hydroquinone unless cytochrome *c*, prepared from some other organ, is added. Measurement of the oxygen uptake and of the volatile base liberated in the presence

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<sup>1</sup> Friedenwald, J. S., and Buschke, W., to be published elsewhere.

and absence of cytochrome *c* should reveal the distribution of adrenalin as substrate between these two enzyme systems. The results of experiments conducted according to this plan have led us to the unexpected conclusion that the actions of these two enzyme systems on adrenalin as substrate are not mutually independent, but that a product of the action of the cytochrome system on adrenalin and on some other hydroquinones is capable of inhibiting the action of amine oxidase on adrenalin and on other similar amines.

### *Technique*

Mucosa from rabbit intestine was used as a source of cytochrome oxidase and amine oxidase. The small intestine of one rabbit was split open and washed with cold tap water. The superficial layer of the mucosa was scraped off with a blunt spatula, ground in a mortar, and suspended in dialyzing tubes. After dialysis for 2 days in the ice box against frequent changes of distilled water and with frequent vigorous mixing of the contents of the tubes, the suspension was emptied into a mortar, ground thoroughly once more, made up to 50 cc. with distilled water, and with enough phosphate buffer of pH 7.4 to make the phosphate concentration 0.2 M. In most experiments we used 1 cc. of this suspension per Warburg vessel, adding 1 more cc. of phosphate buffer of the same concentration and pH. The substrate solutions were neutralized before addition.

Pure polyphenoloxidase was obtained in the form of a concentrated solution from Mr. Stanley Lewis in the laboratory of Professor J. M. Nelson of Columbia University.

Cytochrome *c* was prepared according to the method of Keilin and Hartree (5) and was stored as acetone-precipitated dry powder.

Oxygen uptake was measured by the Warburg manometric method. Volatile base was determined after incubation and measurement of oxygen uptake as follows: The contents of the manometric flasks were acidified with trichloroacetic acid and the precipitated protein separated in a centrifuge. The supernatant fluid was poured into Erlenmeyer flasks, 1 gm. of sodium carbonate was added, and by bubbling through a lively stream of air the volatile base was carried over into wide centrifuge tubes containing 5 cc. of saturated boric acid with 2 drops of brom-cresol green solution as indicator. After 6 hours the stream of air was interrupted and the base titrated with 0.01 N HCl. Further bubbling for another 6 hours showed that no more base could be removed. The method does not permit distinction between ammonia and methylamine.

### *Results*

Suspensions of intestinal mucosa prepared as outlined above have almost no oxygen uptake unless substrate is added. On addition of adrenalin a

rapid oxygen consumption can be measured (Table I). This oxygen uptake is uninfluenced by the addition of cyanide but is completely suppressed on addition of benzedrine. It is, therefore, wholly attributable to the action of amine oxidase on adrenalin. If cytochrome *c* is added, the oxidation of adrenalin is more rapid, and only a small decrease occurs on

TABLE I

*Oxidation of Adrenalin by Intestine Mucosa Preparation*

5 mg. of adrenalin per sample.

Benzedrine, 2.5 mg.	Cytochrome <i>c</i> , 0.25 cc., 1 per cent solution	Oxygen uptake after 30 min.
		<i>c.mm.</i>
—	—	91
+	—	18
—	+	180
+	+	155

TABLE II

*Oxidation of Catechol, Tyramine, and Hydroquinone + Tyramine by Intestine Mucosa Preparation*

Sample	Tyramine, 10 mg.	Catechol, 2.5 mg.	Cytochrome <i>c</i> , 0.25 cc., 1 per cent solution	Oxygen uptake after 20 min.
				<i>c.mm.</i>
A	+	—	—	67
B	+	—	+	77
C	+	+	—	71
D	+	+	+	64
E	—	+	—	6
F	—	+	+	63

Hydroquinone, 2.5 mg.				
	+	—	+	89
	—	+	+	115
	+	+	+	110

addition of benzedrine.<sup>2</sup> Since the presence of cytochrome *c* does not interfere with the action of the amine oxidase on tyramine nor with the inhibition of the amine oxidase by benzedrine, this experiment indicates

<sup>2</sup> High concentrations of benzedrine inhibit both the amine oxidase and the cytochrome oxidase. There is, however, a range of concentration in which only the amine oxidase is inhibited. The precise location of this concentration range seems to vary for different enzyme preparations and must be determined for each preparation in separate experiments.



that when adrenalin is being attacked by the cytochrome system the amine oxidase becomes inactive.

In a similar experiment the two enzyme systems were supplied with two separate substrates, *p*-hydroquinone or catechol for the cytochrome system, tyramine for the amine oxidase (Table II). In the absence of cytochrome *c* only tyramine is oxidized. In the presence of cytochrome *c* plus a hydroquinone the quinone oxidation accounts for the whole oxygen uptake irrespective of whether tyramine is also present or not.

Although the preceding experiments made it probable that the amine oxidase is inactivated by the action of cytochrome oxidase on a hydroquinone, it seemed desirable to obtain more direct evidence on this point. Several samples of the cell suspension which contained both enzymes were

TABLE III

*Tyramine Oxidation by Intestine Mucosa Preparation, after Preceding Oxidation of Catechol by Cytochrome Oxidase System*

Preliminary period, 5 mg. of catechol, 0.25 cc. of 1 per cent solution of cytochrome *c*; concluding period, 1 mg. of cyanide, 10 mg. of tyramine.

Sample	Cytochrome <i>c</i>	Catechol	Preliminary period, oxygen uptake after 20 min.	Tyramine	Concluding period, oxygen uptake after 20 min.
			<i>c.mm.</i>		<i>c.mm.</i>
A	—	—	10	+	55
B	+	—	11	+	57
C	—	+	12	+	72
D	+	+	75	+	27
E	+	+	78	—	17

placed in Warburg flasks. In certain samples the cytochrome oxidase was activated by the addition of cytochrome *c*; in other samples no cytochrome *c* was added and the cytochrome oxidase system remained inactive. Catechol was added as substrate and the samples incubated for a brief period. This preliminary incubation period was terminated by the addition of cyanide and tyramine, simultaneously inhibiting further action of the cytochrome oxidase and providing a substrate for the amine oxidase. It can be seen from Table III that in those samples (A, B, C) in which the cytochrome system was kept inactive in the preliminary period the amine oxidase exhibited full activity after addition of cyanide and tyramine. In the sample in which the cytochrome oxidase system was active in the preliminary period, Sample D, the amine oxidase showed a greatly decreased activity in the second period. The values in Samples C and D have to be corrected for catechol autoxidation by subtraction of Sample E. The data

are from one of three similar experiments which were in complete agreement.

These results were duplicated by an experiment in which polyphenol-oxidase was used as the inactivating oxidizing system instead of cytochrome oxidase. This experiment was carried out in order to see whether the inactivation of the amine oxidase would take place only if the two enzyme systems were in close spatial proximity, perhaps bound to the same structures of the cells, or whether extracellular systems would exhibit a similar effect. To the usual cell suspension, polyphenoloxidase solution was added together with catechol as substrate. No cytochrome *c* was added. Again a preliminary incubation period was concluded by addition of cyanide and tyramine and the oxygen uptake recorded. The results in Table IV show, in complete agreement with the previous experiment, that the action

TABLE IV  
*Tyramine Oxidation by Intestine Mucosa Preparation, after Preceding Oxidation of Catechol by Polyphenoloxidase*

Preliminary period, 0.05 cc. of polyphenoloxidase solution, 5 mg. of catechol; concluding period, 1 mg. of cyanide, 10 mg. of tyramine.

Polyphenoloxidase + catechol	Preliminary period, oxygen uptake after 20 min.	Tyramine	Concluding period, oxygen uptake after 20 min.
	<i>c.mm.</i>		<i>c.mm.</i>
—	18	—	2
—	15	+	59
+	174	+	21
+	182	—	14

of the amine oxidase is abolished in Sample C to which the cyanide-sensitive polyphenoloxidase had been added. The value in Sample C has to be corrected for catechol autoxidation by subtraction of Sample D.

The same type of experiment with adrenalin as substrate for the polyphenoloxidase or cytochrome oxidase does not yield on subsequent mixing with tyramine and cyanide any inhibition of the amine oxidation of tyramine. The same failure of inhibition of the amine oxidase is found when adrenalin itself is used as substrate for both systems in *succession*. Contrasting these results with the experiments reported above on the inactivation of the amine oxidase by *simultaneous* activity of the cytochrome system on adrenalin, we concluded that the inactivation of the amine oxidase by the oxidation product of adrenalin is transitory and is due to a transitory intermediate oxidation product of adrenalin. The existence of such intermediate oxidation products preceding the formation

of adrenochrome has recently been reported (6), and is further supported by some experiments in this laboratory which will be reported elsewhere.<sup>1</sup>

A further test of the correctness of the interpretation of these experiments is achieved by comparing the oxygen uptake with the ammonia production. No ammonia results from the quinone oxidation but between 1 and 2 molecules of ammonia or methylamine are produced for each molecule of oxygen consumed when the amine oxidase acts.<sup>3</sup> Table V shows the result of experiments in which cytochrome oxidase of the tissue was activated by addition of cytochrome c. Without substrate there was a small oxygen uptake and a small  $\text{NH}_3$  production. When tyramine was added, a large and approximately equal increase in  $\text{O}_2$  uptake and  $\text{NH}_3$  production was found. With tyramine and catechol simultaneously pre-

TABLE V

*Oxygen Uptake and Ammonia Production by Intestine Mucosa Preparation*

Substrates, 10 mg. of catechol, 5 mg. of tyramine, and catechol + tyramine; 0.5 cc. of a 1 per cent solution of cytochrome c.

	Oxygen uptake after 90 min.	Ammonia production after 90 min.	
		Uncorrected	Corrected for $\text{NH}_3$ production by tissue alone
	micromoles $\text{O}_2$	micromoles $\text{NH}_3$	micromoles $\text{NH}_3$
Tissue suspension .....	1.0	2.5	
“ “ + tyramine.....	8.0	14.2	11.7
“ “ + catechol.....	12.1	2.6	0.1
“ “ + tyramine + catechol..	12.8	5.1	2.6

sented to the tissue, the  $\text{O}_2$  uptake is the same as that with catechol alone, while the  $\text{NH}_3$  production from the tyramine is 80 per cent inhibited. Similar experiments were carried out with cobefrin (3,4-dihydroxyphenylpropanol amine hydrochloride) and tyramine as substrate pair and with adrenalin alone (Table VI). In both cases the results were in agreement with those just described.

A concluding series of experiments was carried out in order to obtain information in regard to the mechanism of the inactivation of amine oxidase. The detection of an oxidizable group in this enzyme, the integrity of which would prove to be essential for its full activity, would not only make the inactivation intelligible but also contribute to the knowledge of the properties of the enzyme as such. Preliminary experiment showed that the

<sup>3</sup> The chief oxidation product of amine oxidase action under the conditions of our experiments appears to be the carboxylic acid rather than the aldehyde. For the production of the latter  $\frac{1}{2}\text{O}_2$  is required per  $\text{NH}_3$  produced.

TABLE VI

*Oxygen Uptake and Ammonia Production by Intestine Mucosa Preparation*  
 Substrate, 5 mg. of adrenalin; 0.5 cc. of a 1 per cent solution of cytochrome c.

	Oxygen uptake after 60 min.	Ammonia production after 60 min.	
		Uncorrected	Corrected for NH <sub>3</sub> production by tissue alone
	micromoles O <sub>2</sub>	micromoles NH <sub>3</sub>	micromoles NH <sub>3</sub>
Tissue suspension .....	1.4	3.1	
" " + adrenalin.....	7.6	16.0	12.9
" " + " + cyto- chrome c.....	9.7	7.5	4.4

TABLE VII

*Effect of Mercurials upon Activity of Amine Oxidase of Intestine Musoca Preparation*  
 10 mg. of tyramine hydrochloride; mercurials 30 micromoles per vessel.

	Oxygen uptake after 20 min.
	c.mm.
Tyramine.....	76
" + bis- <i>p</i> -tolylmercury .....	77
" + <i>p</i> -chloromercuric benzoate .....	11
" + phenylmercuric hydroxide .....	12

TABLE VIII

*Partial Inactivation of Amine Oxidase by Mercurials, and Recovery of Activity by Addition of Glutathione or Cysteine*

1 mg. of cyanide, 5 mg. of tyramine, 10 mg. of glutathione, 10 mg. of cysteine, 30 micromoles of mercurials.

Substances added to intestine mucosa preparation	Oxygen uptake during 20 min.
	c.mm.
Tyramine.....	90
Glutathione .....	8
Tyramine + glutathione .....	104
" + chloromercuric benzoate .....	57
Chloromercuric benzoate + glutathione.....	9
Tyramine + chloromercuric benzoate + glutathione.....	115
Cysteine.....	12
Tyramine + cysteine .....	105
" + phenylmercuric hydroxide .....	59
Phenylmercuric hydroxide + cysteine.....	12
Tyramine + phenylmercuric hydroxide + cysteine.....	112

activity of amine oxidase could be abolished by treatment with iodine, with methylene blue, and also by prolonged incubation with iodoacetate.<sup>4</sup> Therefore, we set out to test with more specific reagents whether sulfhydryl groups were involved in the inactivation. We used for these tests those organic mercurial compounds which have been used by Hellerman and Perkins (8) in extensive studies of other enzymes.

Of the three mercurials tested one (bistoluymercury) does not react with sulfhydryl groups. This substance produced no inhibition of the amine oxidase (Table VII). The two other compounds (chloromercuric benzoate and phenylmercuric hydroxide) react readily with SH groups and markedly inhibit the amine oxidase. The inhibitory effect was almost complete immediately after addition of the mercurial inhibitors.

In the course of studies on the inhibiting action of mercurials, we found that cyanide in small concentration considerably reduces the inhibitory effect of these compounds. The explanation of this interesting phenomenon does not concern us here, but the action of cyanide proved useful in our studies in that it made possible the demonstration that the inhibitory effect of the mercurials on the amine oxidase could be reversed by the addition of glutathione (Table VIII). In the absence of cyanide the inhibition produced by the mercurials was only very slightly reversed by the addition of sulfhydryl compounds.

#### DISCUSSION

The inactivation of certain enzymes by quinones is not new. In fact a large number of enzymes have been shown to be susceptible to reversible oxidative inactivation by various agents. In respect to some of these enzymes, there is evidence of varying strength, indicating that the group in the enzyme which is attacked by the oxidizing agent is probably a sulfhydryl (9). In this connection the inactivation of amine oxidase by the simultaneous enzymatic oxidation of uric acid reported by Zeller (10) should be recalled.

The present study reveals a possible physiological mechanism by which reversible inactivation of some sulfhydryl enzymes may be achieved. It would be attractive to speculate on the enzyme system which may possibly be susceptible to this mode of inactivation. For instance, the sensitivity of triose phosphate dehydrogenase, which is also inhibited by iodoacetate (11), methylene blue (12), and by oxidizing agents including quinones (13), could give our observations significance for the problem of the Pasteur effect. We have as yet been unable to obtain any evidence that such in-

<sup>4</sup> Kohn (7) failed to obtain inhibition of amine oxidase with iodoacetate, possibly because of too short an incubation period in his experiments. At least 2 hours exposure of the enzyme to iodoacetate are required.

activations are part of the physiological or pharmacological effects of adrenalin. Furthermore we have no evidence that physiological concentrations of adrenalin would suffice to produce such inhibitions. Experimental results obtained on ground and dialyzed tissue need not be a true picture of what can happen in the undisturbed organ and our results provide no basis for deciding which of the many oxidizable components of a living cell would compete most favorably for a locally produced oxidizing agent derived from adrenalin. Without any assumptions regarding the tissue component that may be oxidized by this reaction, our experiments still provide grounds for concluding that the reaction products resulting from the quinone oxidation of adrenalin can act as intracellular oxidizing agents. A possible explanation of the rôle of the cyanide-sensitive oxidase in the pharmacology of adrenalin is thus provided.

#### SUMMARY

The oxidation of adrenalin and certain other quinones by the cytochrome oxidase system or by polyphenoloxidase results in the production of substances capable of inhibiting amine oxidase. The active agent is not adrenochrome but some unstable precursor. The mechanism of inhibition appears to consist in the oxidation of a sulfhydryl group in the amine oxidase.

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# THE EFFECT OF GRAMICIDIN AND TYROCIDINE ON BACTERIAL METABOLISM

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Most of the common antiseptics, heavy metals, chlorine compounds, phenolic compounds, detergents, etc., exert an inhibitory effect upon the metabolism of the susceptible microbial species. There are, however, a few substances which, although possessing marked bacteriostatic activity, do not appear to interrupt respiratory processes. This is particularly true of the sulfonamides. Cultures of *Bacillus brevis* have yielded two crystalline polypeptides, gramicidin and tyrocidine, which are endowed with antibacterial activity but differ in many other biological and chemical properties (1, 2). It will be shown in the present paper that one of these substances, tyrocidine, inactivates completely the oxidation-reduction systems of susceptible cells. In this respect tyrocidine behaves like common antiseptics, and perhaps especially like the cationic detergents, which it resembles in several respects. The other substance, gramicidin, causes on the contrary only a limited injury to the bacterial cells and may either stimulate or depress some of their metabolic functions, according to the composition of the medium in which the test is carried out.

## EXPERIMENTAL

Whereas tyrocidine is toxic for all groups of bacteria, gramicidin is entirely inactive against Gram-negative bacilli (1, 2). For a comparative study of the effect of the two compounds, we have therefore selected strains of Gram-positive cocci which are susceptible to both of them. One, *Staphylococcus aureus* (Strain 40-2B), was isolated from a case of bovine mastitis; the other, *Streptococcus hemolyticus* Group D (Strain H69D), was kindly supplied by Dr. R. C. Lancefield. Although these two strains are not the most highly susceptible to gramicidin and tyrocidine, they offer the advantage of growing readily and abundantly in ordinary bacteriological media, and of representing two fairly different types of cellular metabolism. The streptococcus culture gives rise predominantly to a lactic acid fermentation, whereas aerobic mechanisms play a larger part in the metabolism of the staphylococci.

The bacterial cultures were grown for 18 hours in meat infusion-peptone broth and the cells were separated by centrifugation and washed in water.



Washing often resulted in a marked decrease in metabolic activity and in particular decreased the ability of the cells to exhibit stimulation of metabolism upon the addition of gramicidin. It was found that systems of greater and more dependable metabolic activity were obtained when, following separation from the culture medium, the cells were incubated in a neutral phosphate buffer containing glucose, and separated by centrifugation just prior to use. This procedure was followed in some of the experiments reported.

The gramicidin and tyrocidine hydrochloride (3) were kept as stock solutions containing 5 mg. per cc. in alcohol. Since ethyl alcohol is readily oxidized by staphylococcus, it was often found desirable to use tertiary butyl alcohol, a completely inert substance, as the solvent. The alcoholic solutions were diluted in distilled water. As substrates glucose and ethyl alcohol were used in 0.5 to 1.0 per cent concentration; the nature of the results was not affected by varying the concentration.

Oxygen uptake was measured by the conventional technique in the Warburg respirometer; the reaction was carried out at 38°; CO<sub>2</sub> was absorbed by 20 per cent NaOH in the center cup. The vessels had a volume of about 8 cc. and contained from 1.2 to 1.5 cc. of total fluid.

Cell suspensions of different densities were used in different experiments. In general, it was arranged that the cell suspension be such as to give an oxygen uptake of 50 to 150 microliters per hour, per manometer vessel.

Fig. 1 illustrates the effect of different amounts of gramicidin and tyrocidine on the oxygen uptake of staphylococci resuspended in a solution containing glucose and potassium phosphate buffer at pH 7.4. In this experiment low concentrations of Mg<sup>++</sup>, Mn<sup>++</sup>, and Ca<sup>++</sup> ions were provided; since no effect of these ions has been observed, they were not introduced in the other experiments reported here.

It is seen that, immediately upon the addition of gramicidin, there was an increase in the rate of oxygen uptake which lasted for several hours and was independent of the amount of gramicidin used. On the contrary, the addition of 40  $\gamma$  of tyrocidine caused a complete inhibition of oxygen uptake; no stimulation was observed with 1  $\gamma$  of the substance.

Analogous results were obtained when ethyl alcohol was substituted for glucose. The data presented in Table I illustrate especially that, even when used in excess, gramicidin always caused a stimulation of oxygen uptake, whereas only inhibition could be observed with tyrocidine. The stimulating effect of gramicidin takes place over a wide range of pH, with an optimum around neutrality (Table II); it is influenced not only by the pH of the medium, but also by the nature of the buffer. In citrate buffer at pH 6.7, for instance, gramicidin caused an immediate decrease of oxygen uptake by staphylococci resuspended in 1.0 per cent alcohol; on the other

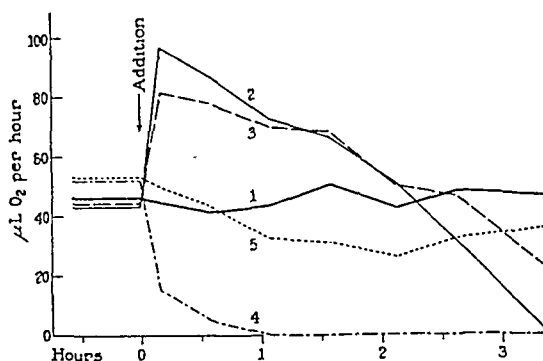


FIG. 1. Rate of oxygen uptake by *Staphylococcus aureus* acting on glucose at 38°. The abscissa represents time; the ordinate, oxygen uptake per  $\frac{1}{2}$  hour by 1.4 cc. of suspension containing the cells from 2 cc. of broth culture, and 10 mg. of glucose. Curve 1, control; Curve 2, 40  $\gamma$  of gramicidin added at zero time; Curve 3, 1  $\gamma$  of gramicidin added; Curve 4, 40  $\gamma$  of tyrocidine hydrochloride added; Curve 5, 1  $\gamma$  of tyrocidine hydrochloride added. Medium, 0.07 M potassium phosphate buffer at pH 7.4 containing (molal concentration  $\times 10^4$ )  $Mg^{++} = 5$ ,  $Mn^{++} = 5$ ,  $Ca^{++} = 6$ ,  $Na^+ = 11$ ,  $SO_4^{--} = 10$ ,  $Cl^- = 70$ .

TABLE I

*Effect of Gramicidin and Tyrocidine on Oxygen Uptake by Staphylococcus aureus Acting on Ethyl Alcohol*

Medium, 0.07 M potassium phosphate, 0.08 M sodium chloride.

Amount of agent per 1.2 cc. final volume	$\frac{Q_{O_2} \text{ with agent}}{Q_{O_2} \text{ without agent}} \times 100$			
	Before addition	Time after addition of agent		
		0-30 min.	60-90 min.	120-150 min.
100 $\gamma$ gramicidin.....	101	125	100	85
30 " ".....	97	122	100	75
10 " ".....	108	117	104	85
3 " ".....	101	110	107	90
1 " ".....	97	124	114	97
0.3 " ".....	107	119	119	112
100 " tyrocidine hydrochloride .....	100	10	0	0
30 " " ".....	96	20	10	0
10 " " ".....	95	55	60	50
3 " " ".....	94	96	91	65
1 " " ".....	97	100	94	80
0.3 " " ".....	97	100	96	85
Control, actual oxygen uptake in cc. per 30 min.....	39	37	27	20

hand, when potassium phosphate at pH 6.7 was present in the same citrate solution, gramicidin caused a stimulation of oxygen uptake.

Other ionic components of the medium also influence the response of the cells to gramicidin. For instance, stimulation of oxygen uptake is barely detectable, or altogether fails, in the absence of potassium, although it is readily observed in the absence of sodium (Table III). Ammonium chloride also exerts a striking action; in the presence of small amounts of this substance gramicidin causes marked depression of oxygen uptake by staphylococcus when the conditions are otherwise favorable for stimulation.

Stimulation of the metabolism of staphylococcus can also be observed when, instead of oxygen uptake, lactic acid production (colorimetric determination (4)) is used as an index of metabolic activity.

TABLE II

*Effect of pH of Medium on Stimulation of Staphylococcus Metabolism by Gramicidin*  
Gramicidin added, 20  $\gamma$  per 1.1 cc. of final volume.

0.09 M potassium phosphate buffer	$\frac{Q_{O_2} \text{ with gramicidin}}{Q_{O_2} \text{ without gramicidin}} \times 100$			
	Before addition	Time after addition of gramicidin		
		0-30 min.	60-90 min.	120-150 min.
pH				
7.8	105	134	119	70
7.4	103	171	199	114
7.0	85	196	210	116
6.6	97	195	177	116
6.2	100	142	117	80
5.8	80	99	50	25

The difference in the mode of action of gramicidin and tyrocidine is well illustrated by comparing the effect of these two substances on the oxygen uptake and acid production by *Streptococcus hemolyticus* Group D. It is seen in Table IV, for instance, that the rate of acid production is increased by gramicidin when the cells are resuspended in a glucose solution containing potassium phosphate, whereas the rate is decreased when the system is buffered with sodium phosphate. In each case, the rates remained uniform as long as glucose was present in the medium and the total acid produced was the same.

Tyrocidine always caused a decrease or complete cessation of acid production by streptococcus in analogous experiments.

In a number of qualitative or semiquantitative experiments, the reducing ability of cell suspensions was determined by observing the reduction of methylene blue or of ferric chloride. In the latter case, ferric chloride

(0.0002 M) and  $\alpha$ ,  $\alpha'$ -bipyridine (0.0004 M) were added directly to the cell suspension; under these conditions, the production of the red complex between  $\alpha$ ,  $\alpha'$ -bipyridine and ferrous ions served as an index of reducing

TABLE III

*Effect of Various Ions on Staphylococcus Metabolism in Presence of Gramicidin*

Gramicidin added, Experiment A, 8  $\gamma$  per 1.4 cc. of final volume; Experiment B, 20  $\gamma$  per 1.2 cc. of final volume; Experiment C, 20  $\gamma$  per 1.2 cc. of final volume.

Experiment	External medium			$\frac{Q_{O_2} \text{ with gramicidin}}{Q_{O_2} \text{ without gramicidin}} \times 100$			
	Buffer and salt	NH <sub>4</sub> Cl	pH	Before addition	Time after addition of gramicidin		
					0-30 min.	60-90 min.	120-150 min.
A	0.02 M Na citrate	0	6.7	98	25	10	15
	0.02 " " " + 0.09 M K phosphate	0	6.7	99	165	125	60
B	0.06 M Na phosphate + 0.08 M NaCl	0	7.4	106	100	60	35
	0.06 " " " + 0.08 " KCl	0	7.4	91	115	65	45
	0.06 " " " + 0.08 " NaCl	0	6.7	100	107	55	45
	0.06 " " " + 0.08 " KCl	0	6.7	85	125	65	55
C	0.08 " K " + 0.08 " NaCl	0	7.4	94	120	75	55
	0.08 " " " + 0.08 " "	0.003	7.4	103	91	70	70
	0.09 " " " "	0	6.7	93	120	55	50
	0.09 " " " "	0.003	6.7	99	35	35	20

TABLE IV

*Effect of Gramicidin on Acid Production by Streptococcus hemolyticus (Group D) Acting on Glucose*

The time indicated is that elapsed after the addition of glucose. Gramicidin, 1.5  $\gamma$  per cc. of bacterial suspension.

Medium	N/70 acid produced after			
	1 hr.	3 hrs.	5½ hrs.	8 hrs. (final)
0.035 M K <sub>2</sub> HPO <sub>4</sub> .....	0.30	0.92	1.84	2.44
0.035 " " + gramicidin.....	0.44	1.33	2.26	2.51
0.035 " Na <sub>2</sub> HPO <sub>4</sub> .....	0.38	1.12	2.10	2.42
0.035 " " + gramicidin.....	0.31	0.98	1.76	2.45

action. Again it was found that tyrocidine always inhibits the reduction of methylene blue and ferric ions, whereas gramicidin greatly stimulates the reduction of Fe<sup>+++</sup> in potassium phosphate buffers.

Henle and Zittle (5) observed that tyrothricin stimulates oxygen uptake

and glycolysis by spermatozoa. Tyrothricin is a mixture containing approximately 10 to 20 per cent gramicidin and 40 to 60 per cent tyrocidine. The data presented in Table I allow one to deduce that, over a narrow range of concentration of tyrothricin, it should be possible to observe stimulation by the gramicidin without appreciable effect of the tyrocidine component.

#### SUMMARY

Tyrocidine inhibits metabolic reactions of *Staphylococcus aureus* and *Streptococcus hemolyticus* (Group D). The degree of inhibition is directly related to the concentration of the inhibitor.

The effect of gramicidin on these same metabolic functions is greatly influenced by the composition of the medium in which the reaction is carried out. In the presence of phosphate and potassium ions, for instance, there is observed a prolonged stimulation of metabolism which is independent of the amount of gramicidin present. In the presence of ammonium ions, gramicidin causes a depression of oxygen uptake by staphylococcus under conditions otherwise favorable for stimulation.

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# QUANTITATIVE DROP ANALYSIS

## XVII. GASOMETRIC DETERMINATION OF AMINO NITROGEN\*

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In a study of enzymic hydrolysis in small samples, either as a histochemical problem or in the study of the metabolism of tissues in culture *in vitro*, it is frequently desirable to analyze for very minute amounts of amino nitrogen. The determination of this constituent in samples containing as little as 1  $\gamma$  has been successfully performed by use of the formol titration method (1, 2). While titrations in the presence of formaldehyde are definitely useful in obtaining comparative results, in analyzing solutions of single amino acids, and in following changes in amino nitrogen content, *e.g.* during hydrolysis, the method is defective as a means of analyzing mixtures of amino acids because of the great dependence of the recovery on the pH end-point chosen. Any particular end-point may give an average recovery which is widely different from the theoretical, and analyses of the same material may yield variable results which depend on this factor alone.

In this respect, the gasometric method of Van Slyke (3) is definitely superior, because it is possible to choose a single set of analytical conditions which will yield recoveries varying not more than 2 to 3 per cent from the theoretical with all of the tested amino acids except proline, hydroxyproline, and lysine. Average recoveries from mixtures of amino acids should then closely approximate complete recovery under these conditions.

The design of the Van Slyke amino nitrogen apparatus as well as that of the manometric apparatus of Van Slyke and Neill (4), which may also be employed for amino nitrogen determination, is clearly not suitable for use with gas volumes of 1 or a few microliters. For this purpose a simple apparatus was devised and used to determine amounts of amino nitrogen as small as 0.5  $\gamma$ . It could be shown that the precision and recovery with this apparatus were essentially the same as with the common larger scale method. This is a matter of additional interest because it represents the first gasometric apparatus so far designed for application to this very small scale of analysis.

### EXPERIMENTAL

*Apparatus*—The apparatus is shown in Fig. 1. It consisted essentially of a reaction chamber *RC* of about 5 ml. capacity, connected with a cali-

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brated measuring capillary  $C$ , filling funnels  $R_1$  and  $R_2$ , connected through the 2-way stop-cock  $S_2$ , and a mercury leveling bulb  $L$  connected to the reaction chamber through the stop-cock  $S_1$ . The measuring capillary  $C$  which was closed with a third stop-cock  $S_3$  was of 1 mm. bore through its lower half and of 0.5 mm. bore through its upper half; it was about 20 cm. in length over the calibrated portion. Stop-cock  $S_2$  was a 1 mm. bore 2-way type with 1 mm. bore side arms. The large funnel  $R_1$  had a capacity of slightly more than 5 ml.,  $R_2$  about 0.5 ml. The entire apparatus was mounted on a wooden back which was suspended freely from a pivot  $P$

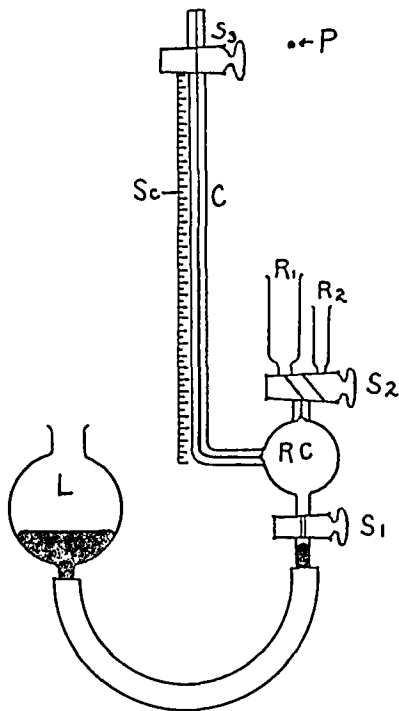


Fig. 1. Apparatus for nitrogen determination

to allow shaking and which could be removed and laid flat when desired. Shaking could be performed by hand or the mounting so arranged as to make use of a small motor.

*Reagents*—Although claims have been advanced for improved types of reagents (5), those of the original Van Slyke method were found to yield excellent results. They were as follows: (a) sodium nitrite, 800 gm. per liter; (b) glacial acetic acid; (c) alkaline permanganate solution, consisting of 10 per cent sodium hydroxide solution saturated with potassium permanganate.

*Procedure*—The capillary  $C$  was filled with water and the stop-cock  $S_3$  closed. The stop-cock  $S_2$  was opened to connect the reaction chamber with the small filling funnel,  $R_2$ . The leveling bulb was raised until mercury just entered  $R_2$  and the stop-cock was closed. About 0.15 ml. of sodium nitrite solution was placed in  $R_2$  and the leveling bulb  $L$  was lowered until most but not all of the nitrite was drawn into the reaction chamber. About 0.07 ml. of glacial acetic acid was then placed in  $R_2$  and  $L$  again lowered until most of it had been drawn into the reaction chamber. The remaining acid was rinsed into the reaction chamber with about 0.4 ml. of distilled water which had been saturated with carbon dioxide by passing the gas through it for 10 to 15 minutes. This had the effect of lowering the blank by expelling dissolved air and aiding in removal of air subsequently from the acid nitrite reagent. After  $S_2$  had been closed and the leveling bulb lowered, the reagent was allowed to evolve gas for 15 seconds, the gas being then expelled through  $R_1$  after the leveling bulb was raised. This operation was repeated once more to rid the solution of air.

The sample, containing from 0.5  $\gamma$  to 20  $\gamma$  of amino nitrogen, was measured as previously described (2) into the filling funnel  $R_2$ , with a capillary pipette. Care was taken to prevent trapping of any air bubbles while the sample was introduced and the pipette rinsed. The leveling bulb was lowered and the sample introduced into the reaction chamber without admitting any air. The funnel and stop-cock were rinsed free of sample by several additions of about 75 microliters of carbon dioxide-saturated, distilled water, each portion being carefully drawn into the reaction chamber without admitting air.

The apparatus was shaken by hand or by motor at an even, unhurried rate for 5 minutes. The presence of mercury in the chamber facilitated the agitation, thus avoiding the violent shaking usually employed. At the end of the reaction time the mercury was lowered just below the stop-cock  $S_1$  which was closed off.

Slightly more than 5 ml. of the alkaline permanganate reagent was then placed in the funnel  $R_1$ , care being taken that no air was trapped in the capillary. The stop-cock  $S_2$  was then turned to connect this funnel with the reaction chamber, allowing the permanganate to be sucked into the chamber until most of the gas was absorbed. The apparatus was then dismounted and shaken in a horizontal position for 4 to 5 minutes to complete the absorption of the excess nitrous oxide and carbon dioxide. The pressure of the gas in the reaction chamber was then made atmospheric by opening  $S_2$  for a few seconds to connect with  $R_1$  which still contained a little permanganate. The apparatus was rotated to trap the remaining nitrogen bubble at the opening of the measuring capillary  $C$ . By raising the leveling bulb and opening  $S_1$ , the bubble was forced into the capillary. The apparatus was then laid in a horizontal position and  $S_3$  opened to the



atmosphere. The volume of the gas in capillary *C* could then be read in terms of a previous calibration. If the volume was relatively large, it was read in the 1 mm. bore portion of *C*. If it was quite small, it was forced further along the capillary into the 0.5 mm. section where it could be estimated with about 4 times the precision of the larger tube. The temperature and pressure were noted and the volume of nitrogen under standard conditions calculated.

In all cases a blank was determined on a volume of water equal to that of the sample, all other operations being performed in exactly the same

TABLE I  
*Analysis of Amino Acid Solutions*

Amino acid	Sample of nitrogen	No. of determinations	Nitrogen found*	Average recovery	Standard error†	
					$\gamma$	per cent
Glycine .....	10.88	6	11.02 $\pm$ 0.43	101.3	0.218	2.0
	3.38	5	3.26 $\pm$ 0.09	97.9	0.062	1.9
	2.03	5	2.02 $\pm$ 0.04	99.4	0.024	1.1
	0.532	9	0.556 $\pm$ 0.032	104.6	0.014	2.6
Valine.....	10.15	7	10.10 $\pm$ 0.05	99.5	0.020	0.2
Leucine.....	11.33	7	11.08 $\pm$ 0.31	98.1	0.135	1.3
Glutamic acid.....	11.07	5	11.51 $\pm$ 0.45	104.0	0.227	2.0
Arginine .....	10.59	8	10.56 $\pm$ 0.18	99.8	0.081	0.8
	10.34	5	10.36 $\pm$ 0.20	100.2	0.114	1.1
Histidine (free base) ..	11.28	6	11.32 $\pm$ 0.10	100.3	0.052	0.5
" (monohydrochloride).	13.30	5	12.79 $\pm$ 0.53	96.0	0.289	2.2
Tryptophane.....	12.10	8	12.28 $\pm$ 0.24	101.5	0.102	0.8
Tyrosine.....	11.86	5	11.85 $\pm$ 0.14	99.9	0.076	0.6

\* Mean plus-minus mean deviation from the true value.

† Calculated from the formula  $s.e. = \sqrt{\Sigma d^2/n(n-1)}$  where  $d$  = deviation from the true value and  $n$  the number of determinations.

manner as in the analysis. This was applied as a correction to the volume of nitrogen noted in the determination. When a new lot of carbon dioxide-saturated water was used, it was found necessary to redetermine a blank, owing to slight differences in the residual gas of different lots.

The apparatus was cleaned by closing  $S_1$  and attaching a vacuum line with an inserted trap to the capillary at  $S_3$ . With the vacuum on, the apparatus was rinsed with enough oxalic acid solution to remove all manganese dioxide, and then with distilled water. Unusually large amounts of manganese dioxide could be removed by applying suction to  $R_2$  and then rinsing as described.

## Results

Since the experimental conditions of this analytical procedure are not identical with those of the original method (3), it was thought desirable to adjust the time of shaking until essentially the same recoveries were obtained as in the original method, rather than to adopt an arbitrary shaking time and recalculate standard tables. The time chosen, viz. 5 minutes, was found to yield results which were close to those of the original method, and,

TABLE II  
*Gasometric Determination of Lysine Monohydrochloride*

Temperature	Length of shaking	Sample of nitrogen	No. of determinations	Nitrogen found*	Average recovery	Standard error†	
°C.	min.	$\gamma$		$\gamma$	per cent	$\gamma$	per cent
26	5	10.48	8	$7.96 \pm 0.08$	75.9	0.037	0.46
37	5	10.48	6	$9.26 \pm 0.11$	88.3	0.057	0.62
26	5	24.42	4	$17.59 \pm 0.06$	72.0	0.032	0.18
26	6	24.42	4	$18.06 \pm 0.08$	73.9	0.056	0.31
26	7	24.42	4	$18.55 \pm 0.20$	75.9	0.134	0.72

\* Mean plus-minus mean deviation from the mean.

† Calculated as in Table I, except that the deviation from the mean was used rather than the deviation from the correct value.

TABLE III  
*Comparison of Recovery with That of Van Slyke (3)*

Amino acid	Mean recovery	Van Slyke's recovery
	per cent	per cent
Glycine.....	101.3	103
Valine.....	99.5	100.0
Leucine.....	98.1	100.3
Glutamic acid.....	104.0	101.0
Arginine.....	100.0	100
Tyrosine.....	99.9	102
Histidine monohydrochloride .....	96.0	98.5
Lysine.....	75 Ca.	85.2

in addition, constituted a convenient time interval. Except in the case of lysine, the recoveries were quite satisfactory with this time of shaking, as shown in Table I. With this single exception the recoveries ranged from about 96 to 104 per cent with samples of 2  $\gamma$  or above. Somewhat more variation was found with smaller samples, as was to be expected. Most of the common amino acids yielded recoveries of  $100 \pm 2$  per cent.

In the hope of finding conditions under which the recovery of amino

nitrogen of lysine would be more nearly quantitative, the time of shaking and the temperature were varied. The recovery was improved somewhat but not markedly when the shaking time was longer than 5 minutes. The effect of raising the temperature from 26° to 37° was considerably more noticeable, increasing the average recovery from 75.9 to 88.3 per cent, a figure which is still below theoretical. The data are shown in Table II.

In Table III is shown a comparison of the recovery values of this procedure compared with those listed by Van Slyke. Again it is apparent that these minute amounts of amino nitrogen can be analyzed gasometrically with almost the same accuracy as larger amounts determined according to the same chemical principle. The precision and number of data are not sufficient to give this comparison a conclusive significance. Thus the lower results with glycine are probably to be explained as due to incomplete recovery rather than to superiority of the procedure.

The method was tested on protein hydrolysates and found to be applicable with about the same precision as is indicated with pure amino acid solutions. Since the absolute quantities of nitrogen were unknown in these instances, no evaluation of the accuracy or completeness of recovery is possible and the data are omitted.

#### SUMMARY

A modification of the Van Slyke gasometric method for determination of microgram quantities of amino nitrogen is described. It was found possible to determine as little as 0.5  $\gamma$  of nitrogen, and with quantities of 2  $\gamma$  or larger to obtain a precision and accuracy which were closely comparable to the macro and micro gasometric procedures.

The apparatus is somewhat simpler than the conventional type and its manipulation not much more difficult, though some additional precautions are necessary.

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# THE NATURE OF THE PROTEINS OF CELLULAR NUCLEI

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Despite the importance of the nucleus in the functional life of the cell, investigations of the chemistry of nuclei, especially of nuclear proteins, are comparatively rare. A large part of our present knowledge of nucleoproteins (*i.e.*, protamine nucleate and histone nucleate) has been derived from early investigations upon the spermatozoa of fishes (1-4). Other than spermatozoa, the only cells investigated have been avian red blood cells (5) and human pus cells (6) and these investigations were made before the more precise methods for the isolation and analysis of proteins were available.

Methods for obtaining cell nuclei from other tissues in sufficient quantities and adequately pure for subsequent analyses have not been developed until recent years, when two were described, one by Behrens (7), the other by Stoneburg (8). Although the latter method is the simpler of the two, the Behrens method requires no reagents save inert organic solvents, which remove lipids but leave the proteins with minimum alteration, until after the nuclei are separated from other protein-containing portions of the cell.

Whether proteins other than the histones and the protamines are combined with nucleic acid in mammalian cellular nuclei is not known. Further, no information has been available on the nature of the proteins presumably present in the nuclei other than those linked with nucleic acid.

The present investigation is an attempt to ascertain what proteins are found in cellular nuclei in addition to the previously known histones and protamines.

## EXPERIMENTAL

*Preparation of Cytoplasmic-Free Material*—Behrens (7) applied his method for the isolation of cell nuclei to calf heart muscle. The greater connective tissue and lipid content of calf thymus tissue, the high nuclear content of which made it suitable for our investigation, compelled us to make several modifications in the original heart muscle technique. At the date when our investigation was started, Behrens had not published his adaptation of his method to the thymus (9).

In the Behrens technique, there are three principal operations: freezing

\* Material from a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Graduate School, University of Missouri.

the tissue and drying it *in vacuo* while in the frozen state, grinding the dried material to a suitable fineness, then separating the nuclei from other cell constituents in an inert liquid of intermediate specific gravity. The thymus glands were secured from calves within 15 minutes after slaughter. As soon as fatty material could be removed, the glands were cut into small pieces and frozen in a mixture of pulverized solid carbon dioxide and ether in a large evaporating dish. The brittle frozen material was broken into small particles in a large mortar, transferred to a cheese-cloth bag, and suspended, without being thawed, in a desiccator charged with approximately 1 liter of sulfuric acid. The desiccator was placed in a specially prepared insulated container, packed with a calcium chloride-ice mixture, and evacuated. The ice-salt mixture was maintained at a temperature of  $-7^{\circ}$  to  $-12^{\circ}$ . Replacement of the sulfuric acid and evacuation of the desiccator were performed at regular intervals. 5 to 8 days were required to dry the tissue completely under the above conditions. The dried tissue was then extracted in a Soxhlet apparatus with ether to remove the lipids and ground until it passed a 100 mesh sieve (sieve opening, 0.15 mm.).

To obtain the nuclear material, the powdered gland was first freed from connective tissue by sedimenting the heavier cellular fractions several times from pure benzene and discarding the benzene which contained the connective tissue. The remaining cellular powder practically free from connective tissue was centrifuged out of progressively heavier mixtures of carbon tetrachloride with benzene, the cake of material with higher specific gravity being left each time at the bottom of the centrifuge tube. When the proportion of the tetrachloride had been increased until the heaviest fraction of the powder would no longer settle, benzene was then added until the heavier nuclear fraction of the powder began to settle under centrifugation. We found that at this final separation the specific gravity of the suspension liquid stood between 1.345 and 1.350 at  $28^{\circ}$ .

Approximately 175 gm. of thymus gland were used in each separation and yielded from 12 to 15 per cent of a cytoplasm-free nuclear powder when calculated upon the basis of the dry weight of the original tissue.

*Identification of Separated Fraction*—The material separated was distinctive in appearance. Whereas the whole gland powder was nearly white in color and of a fine fluffy texture, the heavier, separated, nuclear fraction was buff-colored and granular.

Behrens based the proof for the freedom of his preparation from other cell constituents primarily upon a glacial acetic acid color reaction. Nuclei, but not cytoplasmic material, turn a dark red color when heated with the reagent. Behrens considers the reaction specific for thymonucleic acid. When the whole gland powder was examined microscopically after application of this test, dark red particles scattered throughout a colorless

background material were observed. On the other hand, the nuclear preparation similarly treated was completely colored, with no uncolored particles apparent.

The basic nuclear stains (basic fuchsin, hematoxylin, methyl green, and acetocarmine) also indicated the absence of any except strongly stained material.

A test for free desoxy sugars (10) was negative, indicating that the nucleic acids were not decomposed by this method.

The final indication that our preparation consisted of nuclei without appreciable contamination was based upon the total nitrogen and phosphorus analyses. Table I shows that nuclear fractions of total nitrogen and phosphorus content which were consistently constant within the limits

TABLE I  
*Analyses of Thymus Nuclei and Calf Heart Nuclei*

	Total nitrogen	Phosphorus
	<i>per cent</i>	<i>per cent</i>
Mixed cytoplasmic material, some nuclei present	12.60	2.15
	12.90	1.92
Whole gland powder, dry and fat-free	14.57	2.27
	14.62	2.32
Thymus nuclei free from other cell constituents	14.48	2.62
	14.47	2.63
	14.46	2.56
	14.45	2.58
	14.44	2.55
Heart muscle nuclei, Behrens (7)	14.24	2.47
	14.48	2.52
	14.20	2.54

of experimental error were yielded by the method of separation, the figures agreeing furthermore with the analyses of beef heart nuclei reported by Behrens.

*Separation of Nuclei into Fractions*—Two distinct fractions of nuclei, the nucleic acid and the accompanying histones or protamines, have been recognized for many years. The contrast between the isoelectric points of these two fractions (2) suggested the propriety of an isoelectric precipitation study of the nuclear substance.

Small samples of the separated nuclear preparation were dissolved in 3 per cent NaOH solution, then used for the subsequent isoelectric precipitation studies.

Three methods for the isolation of the histone were investigated: (1) isoelectric precipitation by acetic acid out of the alkaline nuclear solution;

(2) when HCl or H<sub>2</sub>SO<sub>4</sub> was used to precipitate the other nuclear fractions at their isoelectric points, the histones remained in solution and could be recovered afterwards from the filtrate by precipitation with 70 per cent alcohol; (3) a method based on the solubility of histone in 0.81 per cent HCl (11) and its subsequent precipitation by 70 per cent alcohol in the presence of ammonium chloride.

The third method<sup>1</sup> proved superior to the other two. By it approximately 34 per cent of the original nuclear material was found to be histone. The histone nitrogen was 35.75 to 36.25 per cent of the total nitrogen of the thymus nuclei.

*Nucleic Acid*—Attempts were made to recover the nucleic acid quantitatively by isoelectric precipitation with H<sub>2</sub>SO<sub>4</sub> or HCl. We were never able to obtain quantities that even approached the amount calculated to be present on the basis of the phosphorus analyses. Dialysis experiments indicated definitely that the nucleic acid had been markedly changed during the isolation procedures. The water, inside and outside the collodion dialysis bag, contained a soluble phosphorus compound even after complete precipitation had occurred. Soluble nitrogen compounds were also discovered in the clear solution within the dialysis bag, but no nitrogen was detectable outside the bag.

Since the recovery of the nucleic acid was not quantitatively successful, we calculated the amount present in the thymus nuclei on the basis of their phosphorus analyses. Then using 13.32 per cent (Steudel's (12)<sup>2</sup> and our own analyses of pure preparations of yeast nucleic acid) as the nitrogen content of nucleic acid, we determined by calculation<sup>3</sup> that the nucleic acid contained 31.8 per cent of the total nitrogen of thymus nuclei.

*New Protein Fraction Isolated (pH 5.8 to 6.15)*—Nowhere in the literature has anyone cited the actual isolation of proteins from any type of nuclear tissue other than the basic histones and protamines whose isoelectric points are above pH 8.

Consistently, even though the nuclear material was secured from different calf thymus glands, we always obtained a protein fraction which precipitated at pH 5.8 to 6.15; that is, at a point between the widely separated isoelectric points of the nucleic acid and histone fractions. The new fraction was histone-free and phosphorus analyses indicated the absence of

<sup>1</sup> Higher yields of uncontaminated histone were obtained by this method, as indicated by total nitrogen determination, freedom from phosphorus (nucleic acid), and freedom from other contaminants as shown by solubility tests (2).

<sup>2</sup> Steudel's analyses of thymus nucleic acid and our analyses of pure yeast nucleic acid yielded 13.32 and 13.23 per cent for the nitrogen content and 7.60 and 7.61 per cent for the phosphorus content of these substances respectively.

<sup>3</sup> Assuming that all the phosphorus in thymus nuclei represented nucleic acid.

nucleic acid contamination. This fraction, representing approximately 20 per cent of the nitrogen of the nuclei, was either a new protein or a mixture of several proteins previously unreported, or degradation products of proteins other than histones or protamines.

The new protein fraction when first precipitated was white, but while being dried *in vacuo* darkened to a bluish gray color. The usual qualitative tests ruled out any but traces of iron, while contamination with other metals was excluded by the experimental procedure. Qualitative tests for sulfur were then made and the presence of large quantities of labile sulfur was indicated.

Quantitative estimation of the sulfur showed that the thymus nuclei contained 0.450 to 0.455 per cent sulfur which was bound by a double bond to a carbon atom as indicated by the method of Grote (13). Additional experi-

TABLE II  
*Isolation of Sulfur-Containing Protein*

Fraction pptd. at pH 5.8-6.15				Fraction extracted by 5 per cent NaCl†		
Per cent nuclear material by weight	Total nitrogen*	Sulfur (Stockholm and Koch (14))*	Per cent S of nuclear material	Per cent total nuclear material by weight	Sulfur	Per cent total S of nucleus
	<i>per cent</i>	<i>per cent</i>			<i>per cent</i>	
19.19	14.11	0.599	23.1	8.78	1.31	25.7
19.26	14.00	0.604	23.4	8.78	1.35	25.9
19.34	14.15	0.589	23.0			

\* Based on fat- and moisture-free powder.

† Nitrogen analyses were planned, but the sulfur analyses of this fraction utilized all of the available material. The detailed procedure involved in the preparation of further fractions and other circumstances prevented completion of these analyses at this time. The coagulation temperature of the fraction was 92.5°.

ments by Grote's technique indicated the absence of glutathione and of the C—S—H linkage.

*Isolation of a Sulfur-Containing Protein Moiety by Two Different Methods*  
—The sulfur-containing protein fraction could be isolated by two fundamentally different procedures, each of which yielded the fraction free from histone and nucleic acid.

In the first procedure, 2 gm. samples of the nuclei were dissolved in 60 cc. of 3 per cent NaOH. After the solution had stood for 8 hours at 5°, a gelatinous mixture was produced which was heated in a water bath at 60–70° for 2 hours. Then 5 N H<sub>2</sub>SO<sub>4</sub> was added slowly until pH 5.5 to 6.0 was reached. After being cooled, the solution was centrifuged and the supernatant liquid saved. The latter contains the nucleic acid and the histone fractions. The precipitate was washed with a buffer solution (pH 6.0) and



redissolved in 10 to 15 cc. of 3 per cent NaOH solution. The solution of the protein fraction was then brought to pH 5.5 with 50 per cent acetic acid and 50 cc. of a saturated solution of zinc acetate in formalin were added. The acetate-formalin solution applied to a protein-nucleic acid solution precipitates the protein and retains the nucleic acid in solution. The resulting precipitate was separated by centrifugation, dried with acetone and ether, and then in a vacuum.

In a second method, 2 to 3 gm. samples of the thymus nuclei were extracted with 100 cc. of a 5 per cent NaCl solution in a water bath at 55–60° for 3 hours. The 5 per cent NaCl extract was filtered while hot and the residue thoroughly washed with warm 5 per cent NaCl solution. The filtrate was then dialyzed until free from chlorides and the precipitate recovered by centrifugation and dried as described in the first procedure.

Table II gives the comparative analyses of the fractions isolated by the two procedures.

#### DISCUSSION

The evidence cited indicated rather conclusively that the nuclear fraction of thymus gland tissue prepared by a modification of Behrens' method (7) was essentially free from other cell constituents. We therefore consider the substances isolated by us from this fraction to be those normally found or products derived from those normally found in cell nuclei rather than residues or contaminations remaining from other fractions of the glandular tissue.

Two products, protein in nature, were isolated from the nuclei by two distinct methods, one by an isoelectric precipitation technique, the second involving the solubility of one of the products in 5 per cent NaCl. The 5 per cent NaCl-soluble component constituted but 8.9 per cent of the total nuclear fraction, whereas the fraction precipitated at pH 5.8 to 6.16 constituted approximately 19.2 per cent of the total nuclear fraction. However, in spite of the great difference between the yields of the two products, both contained approximately an equal fraction (23 to 25 per cent) of the sulfur of the total nuclear material. This would indicate that we are concerned with a mixture of proteins or their larger cleavage products rather than with a single substance, and that one of the components contains a high percentage of sulfur and is completely isolated by both methods, while the sulfur-poor or sulfur-free fractions are precipitated only by the isoelectric method.

We are unable at present to account for the location of the remaining 75 per cent of the sulfur in the nuclei. No investigator has suggested that nucleic acid contains sulfur. In human pus nuclei and in bull spermatozoa, Miescher (6, 4) found sulfur compounds which may have been protein in

nature. The histone of the thymus gland contains sulfur when prepared according to the methods of two investigators (15, 16), while in three other preparations the investigators either found no sulfur or failed to test for it (17-19). But so far as we are aware, the presence in thymus histone of any of the known sulfur-containing amino acids has not been reported. Possibly then some protein other than histone is responsible for this residual sulfur. Or, it could be that the residual sulfur is not present in nitrogen-containing compounds and that our extracts represent practically all of the proteins that were present, since these three fractions contained approximately 90 per cent of the total nitrogen of the nuclear material.

Since the literature of the chemistry of nuclei contains no account of substances comparable to ours, no attempt at complete identification can be made until their individual amino acid constitution can be ascertained. However, the extensive experiments of Kato (20) upon the agglutination of mammalian spermatozoa might present a possible clue towards identification. Kato showed the presence of three pH values (isoelectric points), *viz.* 5.6 to 6.8, 2.8 to 3.0, and some value above pH 8.3, at which horse spermatozoa agglutinate suddenly. Kato attributed the agglutinations at the highest and lowest pH values to the presence of protamines and nucleic acid, respectively.

Interestingly, the agglutinations which occurred at pH values of 5.6 to 6.0 were attributed to the albumins and globulins, as these pH values coincided with the zone of flocculation of these proteins as shown by Csonka, Murphy, and Jones (21).

Nevertheless, Kato's experiments upon mammalian spermatozoa showed the existence of three protein fractions precipitable at pH values corresponding to the pH ranges within which the three main fractions of thymus nuclei were precipitated, and indicated that our new sulfur-containing fraction, which is precipitated at intermediate pH values, might be classified as a substance, protein in nature, and possessing the solubility and isoelectric point of a globulin associated with another component not rich in sulfur and not possessing the characteristics of either albumin or globulin.

#### SUMMARY

1. A description has been presented of methods for the preparation of a dry fat-free powder from the thymus gland, which is practically free from all other cell constituents save nuclear material.

2. The isolation of a new sulfur-containing fraction from the protein moiety of the nuclei of the thymus gland has been accomplished by two different methods. This fraction is precipitable isoelectrically at pH 5.8 to 6.2 and is also soluble in 5 per cent salt solution.

3. When precipitated isoelectrically, the sulfur-containing fraction is as-

sociated with other materials of protein nature not rich in sulfur. No further characterization has been made of this additional fraction.

4. Evidence was found for the existence of sulfur in some other compound or compounds, not yet identified.

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# A LACTOBACILLUS ASSAY METHOD FOR *p*-AMINOBENZOIC ACID

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*p*-Aminobenzoic acid has elicited much interest recently because of (a) its antagonistic action toward the activity of sulfanilamide and related drugs, (b) its essential nature in the nutrition of several microorganisms, and (c) evidence of its vitamin activity in several species of higher animals (4). Assay methods for *p*-aminobenzoic acid that combine sensitivity and specificity have not yet been developed. Bratton and Marshall's method (3) for diazotizable amines has been used to follow the isolation of *p*-aminobenzoic acid from yeast (2). A semiquantitative microbiological assay with the bacterium *Clostridium acetobutylicum* was devised by Rubbo *et al.* (8) for the same purpose. The distribution of naturally occurring sulfanilamide inhibitors has been studied (6). Such activity may prove to be correlated with the *p*-aminobenzoic acid content.

While the work reported in this paper was in progress, Isbell (5) reported the growth factor activity of *p*-aminobenzoic acid for *Lactobacillus arabinosus* 17-5. Unpublished results obtained by the present author are in agreement with those published by Isbell. Microbiological assay methods for riboflavin, pantothenic acid, nicotinic acid, and biotin, by means of various species of *Lactobacilli*, are attracting much interest because of their simplicity and rapidity. The fact that with these methods it is possible to use titratable acidity as a measure of growth factor response is of considerable practical importance. The possibility of using *Lactobacillus arabinosus* 17-5 for the microbiological assay of *p*-aminobenzoic acid was therefore investigated.

## EXPERIMENTAL

*Lactobacillus arabinosus* 17-5 was used as a test organism for the assay method described in this paper for *p*-aminobenzoic acid. This bacterium is the same as that used by Snell and Wright (9) in their method for nicotinic acid assay. Cultural details and basal media for the *p*-aminobenzoic acid and nicotinic acid assays are similar in many respects.

**Basal Medium**—The composition of the basal assay medium is given in Table I. The constituents of the medium are prepared as follows:

**Norit-Treated Acid-Hydrolyzed Casein**—100 gm. of vitamin-free casein

(Labco) are hydrolyzed under a reflux for 9 hours with 1000 cc. of 6 *N* hydrochloric acid (10). To remove most of the hydrochloric acid the mixture is then concentrated *in vacuo* to a thick syrup, dissolved in water, and reconcentrated. The syrup is diluted to 900 cc., adjusted to pH 3.0 with concentrated sodium hydroxide, and stirred for 30 minutes with 9 gm. of norit A (Pfanstiehl). The activated carbon is removed by filtration through a Whatman No. 3 filter paper, and the norit treatment and filtration repeated. The filtrate is adjusted to pH 6.8 with concentrated sodium hydroxide, and is diluted to 1000 cc. The hydrolyzed casein is stored in the refrigerator. On standing, precipitation of tyrosine occurs. No difference in results has been noticed whether this precipitate is discarded or whether it is included in the basal medium.

TABLE I  
Composition of Basal Medium

	per cent		p.p.m.
Glucose .....	1.0	Adenine.....	10
Norit-treated acid-hydrolyzed casein .....	0.5	Guanine.....	10
<i>l</i> -Cystine.....	0.01	Uracil.....	10
<i>l</i> -Tryptophane.....	0.01	Thiamine.....	0.1
Sodium acetate ..	0.6	Riboflavin.....	0.2
Potassium monohydrogen phosphate trihydrate .....	0.05	Pantothenic acid....	0.1
Potassium dihydrogen phosphate.....	0.05	Nicotinic acid.....	0.4
Magnesium sulfate heptahydrate .....	0.02	Pyridoxine.....	0.1
Sodium chloride. ....	0.001	Biotin.....	0.0004
Ferrous sulfate heptahydrate ....	0.001		
Manganese sulfate tetrahydrate . ....	0.001		

*l*-Cystine—1 gm. of *l*-cystine is suspended in a small volume of water, 2 cc. of hydrochloric acid are added to dissolve the cystine, and the solution is diluted to 1 liter.

Adenine, Guanine, and Uracil—A solution containing 1 mg. per cc. of each of these chemicals is convenient. 100 mg. of uracil, 124 mg. of guanine hydrochloride, and 174 mg. of adenine sulfate are suspended in a small volume of water, 2 cc. of hydrochloric acid are added, and the mixture heated to solution and then diluted to 100 cc.

Vitamin Supplement—A solution containing 1 mg. of thiamine, 2 mg. of riboflavin, 1 mg. of pantothenic acid, 4 mg. of nicotinic acid, 1 mg. of pyridoxine, and 4  $\gamma$  of biotin per 100 cc. is prepared. Pure, synthetic vitamins are used in all cases except that of biotin. Here crystalline biotin (S. M. A. Corporation) is used. The solution is stored in the refrigerator and is renewed monthly. This is conveniently done by diluting

more concentrated solutions of the individual vitamins, which are stable for longer periods. Exposure of the riboflavin to light should be minimized.

*Inorganic Salts*—These are supplied in two solutions. Solution A contains 25 gm. each of potassium monohydrogen phosphate trihydrate and potassium dihydrogen phosphate per 250 cc. Solution B contains 10 gm. of magnesium sulfate heptahydrate and 0.5 gm. each of sodium chloride, ferrous sulfate heptahydrate, and manganese sulfate tetrahydrate per 250 cc. A few drops of concentrated hydrochloric acid added to Solution B prevent precipitation.

*Glucose*—Several lots of c.p. anhydrous glucose have proved quite satisfactory so far as *p*-aminobenzoic acid blanks are concerned.

Stock solutions, with the exception of the inorganic salt solutions, are stored in the refrigerator. The author has experienced no difficulty with bacteriological contamination, but if this occurs, toluene or a mixture of toluene and chloroform may presumably be used, as they have been successfully used in other *Lactobacillus* methods.

### Procedure

The basal medium is mixed immediately prior to use. To prepare, for example, 1 liter of solution (enough for 200 tubes or twenty assays), 20 gm. of glucose, 12 gm. of sodium acetate, and 200 mg. of *l*-tryptophane are weighed dry and dissolved in the proper amount of water (640 cc.). Then 200 cc. of *l*-cystine solution, 100 cc. of hydrolyzed casein, 20 cc. of the adenine-guanine-uracil mixture, 20 cc. of the vitamin supplement, and 10 cc. each of inorganic Solutions A and B are added. The medium is mixed and adjusted to pH 6.8 with sodium hydroxide.

5 cc. aliquots of the basal medium are dispensed, preferably with an automatic pipette, into Pyrex test-tubes (16 × 150 mm.). These should be fairly uniform in diameter and should preferably be rimless. They may be conveniently handled in wire racks that can be autoclaved.

Appropriate aliquots of the standard *p*-aminobenzoic acid solution and the solutions or suspensions to be assayed are next added and the total volume in each tube made to 10 cc. with distilled water. The tubes are plugged with cotton and autoclaved at 15 pounds of steam pressure for 10 to 15 minutes. The time of autoclaving should be held to a minimum to limit the formation of inhibiting products of unknown nature.

The tubes are cooled, inoculated, and incubated as described later. After incubation the acid produced is titrated with 0.1 *N* sodium hydroxide. Brom-thymol blue (1 drop of 0.4 per cent solution) is satisfactory as an indicator. If the number of tubes is large, or titration must be interrupted or postponed, growth and acid production are stopped by autoclaving the tubes.

The sensitivity of the method necessitates rigid precautions against chemical contamination. Substantial blanks from glassware as ordinarily washed with soap and water have been observed. All equipment with which the basal medium or diluted samples, etc., come in contact should be thoroughly cleaned with an acid cleaning mixture and carefully rinsed. No difficulties with toxicities arising from the use of a dichromate cleaning solution have been noted with this organism.

*The Microorganism*—The stock culture of *Lactobacillus arabinosus* 17-5<sup>1</sup> is carried in yeast extract-glucose-agar stab cultures. Transfers are made monthly, incubated at 30° for 24 hours, then stored in the refrigerator.

For subculturing the organism for inoculum, the basal medium diluted with an equal volume of water to which 1 millimicrogram of *p*-aminobenzoic acid per 10 cc. has been added is dispensed into centrifuge tubes. These are plugged with cotton and sterilized, and if covered with Parafilm may be kept at room temperature for several weeks.

The organism is incubated in this subculturing medium for 24 hours at 30°. The cells are centrifuged and resuspended in the original volume of sterile 0.9 per cent sodium chloride solution. The assay tubes are inoculated from this suspension. This may be done most conveniently by using a sterile 5 cc. syringe with a 22 gage hypodermic needle. 1 drop, equivalent to approximately 0.04 cc., is used per tube. Bacteriological contamination must be avoided by the use of rigidly aseptic technique. The assay tubes are incubated at 30° for 3 days.

*Standard Curve*—The assay range is approximately 0.15 to 0.5 millimicrogram of *p*-aminobenzoic acid per tube. A standard solution containing 10  $\gamma$  per cc. of *p*-aminobenzoic acid is diluted to contain 0.10 millimicrogram per cc. This standard is dispensed in 0, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 cc. aliquots. Two or more replicates are used at each level. A standard curve (Fig. 1) is based on titrations of the acid produced in these tubes.

Aliquots of the material to be assayed are chosen to give titrations falling on the sharply ascending portion of the standard curve. If the approximate *p*-aminobenzoic acid content is unknown, it must be determined by serial dilutions in a preliminary assay. It is desirable to have two or more tubes at each of three aliquot levels represented on the usable portion of the standard curve. The values for the different levels are averaged to give the final assay result.

Assay values calculated from the different levels should agree closely. Any trend of assay values which indicates a variation with size of aliquot

<sup>1</sup> No. 8014, American Type Culture Collection, Georgetown University Medical School, Washington, D. C.

is evidence of the presence of interfering factors, and materials giving such trends will require special investigation. Such trends have rarely been observed with this method.

*Preparation of Samples for Assay*—The method has been applied thus far to water-soluble materials, to water-soluble extracts of natural products, and to yeast suspensions. In some cases the effect of alkaline hydrolysis under steam pressure has been studied, and in many of these cases increased *p*-aminobenzoic acid assays were found. The conditions necessary completely to liberate inactive *p*-aminobenzoic acid without destruction have not been worked out. The samples should be adjusted to approximate neutrality unless this precaution is rendered unnecessary by high dilution.

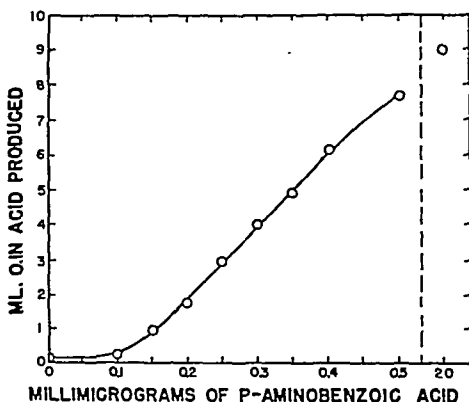


FIG. 1. Response of *Lactobacillus arabinosus* to added *p*-aminobenzoic acid

Preliminary tests of the inactivation of dilute *p*-aminobenzoic acid solutions on autoclaving in the presence of acidic and alkaline conditions have been made. A solution containing 20 millimicrograms of *p*-aminobenzoic acid per cc. in distilled water or in 0.1 N sodium hydroxide was not affected by 30 minutes of autoclaving at 10 pounds of steam pressure. Under the same conditions 1 N sodium hydroxide gave approximately 10 per cent inactivation, while 10 N sodium hydroxide gave only 50 per cent inactivation. Sulfuric acid in concentrations from 0.1 to 10 N gave approximately 30 per cent inactivation. Hydrochloric acid gave similar results. Increased times of autoclaving were relatively more detrimental under acidic than under alkaline conditions. A more concentrated solution containing 1  $\gamma$  of *p*-aminobenzoic acid per cc. was inactivated at approximately the same rate as the solution containing 20 millimicro-



grams of *p*-aminobenzoic acid per cc. under acidic conditions, but under alkaline conditions the more concentrated solution was relatively more stable.

### DISCUSSION

*Reliability of Method*—The reliability of the method is indicated by the excellent duplication of assay values on the same material in different experiments. This reproducibility appears to be similar to that attainable by the other *Lactobacillus* methods, and is of the order of 5 to 15 per cent. Typical examples are given in Table II.

Absence of interfering factors is attested by the agreement of assay values obtained from the different levels of added sample. This agree-

TABLE II  
*Reproducibility of Assays*

Sample	Mean <i>p</i> -amino- benzoic acid content	No. of inde- pendent assays	Standard devia- tion of indi- vidual assays
	$\gamma$ per gm.		per cent of mean
Dried whole egg (commercial)	0.247	7	5
Peptone (Difco)	0.188	3	5
Asparagus juice (dry basis)	1.15	4	13
Yeast extract (Difco)	157	4	4
<i>Torula</i> yeast (fresh basis)	1.74	4	7
Spent <i>Torula</i> culture medium (fresh basis)	0.162	7	12
Biotin concentrate (S. M. A. Corporation, No. 200)	0.184*	5	9

\* Per 20  $\gamma$  of biotin.

ment appears to be superior to that attained in certain other *Lactobacillus* methods. This is not surprising in view of the high dilutions of many materials necessary to bring them within the assay range compared with the dilutions required in other methods. A dilution as high as 1:2,000,000 proved necessary in the case of Difco yeast extract. Increased freedom from interfering factors introduced in the sample thus partially compensates for the increased labor involved in dilutions and in precautions against *p*-aminobenzoic acid contamination. On the other hand, certain biological materials such as blood may contain so little *p*-aminobenzoic acid that direct assays are difficult.

The results in Table III show that the recoveries of *p*-aminobenzoic acid added to biological materials were quantitative within the limits of experimental error.

*Specificity of Method*—The structural simplicity of *p*-aminobenzoic acid arouses considerable interest in the question of the activity of related compounds. Samples of the ortho and meta isomers were found to have 0.00005 and 0.009 per cent, respectively, of the growth-stimulating activity of *p*-aminobenzoic acid. Similar or smaller activities have been found for such other compounds as have been investigated. These activities may be tentatively explained as due to *p*-aminobenzoic acid impurities in the compounds. Proof that the activity is not inherent in these compounds themselves will, however, await purification experiments. The study of specificity is being continued, and the results will be presented elsewhere. It is not unlikely that certain related compounds will be

TABLE III  
*Recovery of p-Aminobenzoic Acid*

Sample	<i>p</i> -Amino- benzoic acid content of sample	<i>p</i> -Amino- benzoic acid added to sample	Total <i>p</i> - aminoben- zoic acid in sample	<i>p</i> -Amino- benzoic acid found	Recovery
	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	per cent
Yeast extract (Difco)	157	240	397	412	104
	157	160	317	316	100
Spent <i>Torula</i> yeast culture medium	0.162	0.150	0.312	0.352	113
	0.162	0.200	0.362	0.348	96
Dried whole egg (commercial)	0.247	0.240	0.487	0.528	108
	0.247	0.240	0.487	0.516	106
Biotin concentrate (S. M. A. Cor- poration, No. 200)	0.184	0.160	0.344	0.344	100
	0.184	0.150	0.334	0.313	94
Standard solution of <i>p</i> -amino- benzoic acid	1.000		1.000	0.992	99
	1.000		1.000	0.982	98
	1.000		1.000	0.979	98
	1.000		1.000	1.037	104

found to possess *p*-aminobenzoic acid activity. Thus Rubbo and colleagues (8) found that the sodium salt, the ethyl ester, and *p*-aminobenzaldehyde possess activity for *Clostridium acetobutylicum* approximately equal to that of *p*-aminobenzoic acid. They also found that the benzoyl derivative, the diethylaminomethyl ester, and *p*-nitrobenzoic acid have approximately one-tenth the activity of *p*-aminobenzoic acid. Of particular interest is the further observation by Rubbo and colleagues that *p*-aminophenylacetic acid has approximately 10 times the activity of *p*-aminobenzoic acid for *Clostridium acetobutylicum*.

*p*-Aminobenzoic Acid Content of Biological Materials—*p*-Aminobenzoic acid contents of miscellaneous biological materials are presented in Table IV. The limited data presented indicate the occurrence of appreciable

TABLE IV  
*p*-Aminobenzoic Acid Contents of Biological Materials

Sample	Water extract or suspension	Sodium hydroxide- treated extract or suspension*
	<i>p.p.m.</i>	<i>p.p.m.</i>
Dried brewers' yeast (No. 1).....	61	59
" " " (" 2).....	11.0	14.6
" " " (" 3).....	6.6	9.3
" autolyzed yeast (Difco).....	7.4	12.0
Autolyzed <i>Torula</i> yeast (fresh basis).....	1.74	2.04
Spent <i>Torula</i> culture medium (fresh basis).....	0.162	0.235
Yeast extract (Difco).....	157	156
Galen "B" (rice bran concentrate).....	2.00	16.2
Dried whole egg (commercial, No. 1).....	0.247	0.30
" " " (" 2).....	0.195	0.238
" " " (Difco).....	0.36	
" egg yolk ".....	0.80	
" " albumin (Difco).....	0.055	
Asparagus juice concentrate (dry basis).....	1.15	2.00
Dried carrots.....	0.178	0.43
" cabbage.....	9.7	13.9
Rice polish concentrate (Labco).....	2.95	9.2
Malt extract (Difco).....	0.74	2.20
Skim milk (No. 1, fresh).....	0.0040	
" " (" 1, slightly sour).....	0.011	
" " (" 1, sour).....	0.019	
" " (" 2, fresh).....	0.0046	0.0043
" " (" 2, sour).....	0.0075	0.0098
Whole blood (ox).....	0.0004	
Beef extract (Difco).....	0.073	0.157
Peptone (Difco).....	0.188	0.204
Neopeptone (Difco).....	0.11	
Proteose-peptone (Difco).....	0.37	
Protone (Difco).....	0.06	
Tryptone ".....	0.43	
Gelatin ".....	0.013	
Nucleic acid (Eastman).....	0.40	0.64
Urine (human, No. 1).....	0.0135	0.35
" ( " " 2).....	0.021	0.49
	<i>γ per γ biotin</i>	<i>γ per γ biotin</i>
Biotin concentrate (S. M. A. Corporation, No. 200).....	0.0092	0.125
" " ( " " " 1000).....	0.0154	
" " ( " " " 5000).....	0.0132	

\* Sample in 1 N sodium hydroxide autoclaved 30 minutes at 13.5 pounds of steam pressure, then cooled, neutralized, and diluted for assay.

amounts of inactive *p*-aminobenzoic acid in certain biological materials, which may be activated by alkaline hydrolysis. The proportion of inactive to total *p*-aminobenzoic acid may be as high as 96 per cent in the case of urine. These results are of interest in view of the observation (6) that a sulfanilamide inhibitor frequently occurs naturally in an inactive form that may be released by hydrolysis or autolysis.

Values for *p*-aminobenzoic acid in biological materials are seldom found in the literature. Rubbo and colleagues (8) isolated as the benzoyl derivative 0.17 part per million of *p*-aminobenzoic acid from moist brewers' yeast. Blanchard (2) isolated as the acetyl derivative 2.7 p.p.m. of *p*-aminobenzoic acid from fresh, plasmolyzed, pressed bakers' yeast. This was 57 per cent of the total diazotizable amines present calculated as *p*-aminobenzoic acid. When the yeast was permitted to autolyze, these values were increased approximately 70 per cent. These values are of the same order as those presented by the author in Table IV.

*Standard Curve*—The standard curve obtained with this method is sigmoid in form. This type of curve is not generally found in the other *Lactobacillus* methods, although on rare occasions the author has observed slight tendencies in this direction in the *Lactobacillus casei*  $\epsilon$  method for pantothenic acid of Pennington *et al.* (7). The "pantothenic acid-less" mutant of *Neurospora crassa* (No. 5531) of Beadle and Tatum (1), when used in the tube growth method (on agar) or when grown on a liquid medium in the presence of graded additions of pantothenic acid, has been observed (unpublished data of the author) to exhibit an exaggerated form of this phenomenon. The cause of this type of response is not apparent but may, perhaps, be due to action of some constituent in the medium in combining or reacting with a portion of the growth factor under consideration, rendering it unavailable to the organism.

Some effort was devoted to a search for factors affecting the form of the standard curve for *p*-aminobenzoic acid. It was unaffected by alterations in the manner of preparation of inoculum or rate of inoculation, by the addition to the basal medium of levels of *o*-aminobenzoic acid, sulfanilic acid, or aniline, which were large in comparison with effective levels of *p*-aminobenzoic acid, or by sundry changes in the medium. The form of the curve varied somewhat with different lots of hydrolyzed casein. The lag phase of the curve was substantially lengthened by the addition of suitable concentrations of sulfanilamide (0.01 to 0.1 p.p.m.) without markedly decreasing the slope of the ascending portion of the curve.

Whatever the cause of this lag in the response of acid production to added *p*-aminobenzoic acid, it apparently does not deleteriously affect the accuracy of the method. This is demonstrated by the reproducibility of assay results. In point of fact the form of the curve is such as to com-

compensate for the somewhat greater variability between replicate assay tubes found with this method.

*Availability of p-Aminobenzoic Acid in Biological Materials*—The questions of preparation of materials for assay and occurrence of *p*-aminobenzoic acid in active and inactive, extractable, and combined forms have been touched but lightly. Undoubtedly each class of material to be assayed should be thoroughly investigated with regard to these points before analytical data are interpreted as representing total *p*-aminobenzoic acid contents.

#### SUMMARY

A rapid and accurate microbiological assay method for *p*-aminobenzoic acid has been developed. It is based on the growth factor activity of *p*-aminobenzoic acid for *Lactobacillus arabinosus* 17-5. Its reliability is indicated by reproducibility of assays, by agreement of assay values obtained with varying levels of added sample, and by recoveries of *p*-aminobenzoic acid added to various samples. The method is very sensitive, the assay range being 0.15 to 0.5 millimicrogram of *p*-aminobenzoic acid. Assay values for a number of biological materials are included. The occurrence of biologically inactive forms of *p*-aminobenzoic acid is indicated. These are activated by alkaline hydrolysis.

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## FERRITIN\*

### I. PHYSICAL AND CHEMICAL PROPERTIES OF HORSE SPLEEN FERRITIN

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In 1937 Laufberger (1) isolated a crystallizable protein from horse spleen which contained over 20 per cent by dry weight of iron. The protein crystallized out readily as a cadmium salt and was stable between pH 4 and 10. Kuhn, Sørensen, and Birkofer (2) confirmed these findings, and from their experiments concluded that ferritin consisted of 54.5 per cent protein, 12.1 per cent nucleic acid, and 35 per cent  $\text{Fe}^{+++}\text{OOH}$ . The protein nature of the compound was corroborated by the analyses of amino acids after hydrolysis. Since there is an iron atom for almost every peptide group, they postulated that each iron atom was attached to each CONH group. (We may remark here that an alternative hypothesis will be suggested presently.) A further study of ferritin, with regard to its chemical, physical, crystalline, and magnetic properties will be the subject of a series of papers, of which this first one is concerned mainly with the preparation of ferritin and some of its physicochemical properties.

The spleen of a normal horse is of a dark brown color, this being due in part to its large content of brown-black hemosiderin granules and in part to the brown color of ferritin. Temporarily one may classify the non-hematin iron of the spleen into three fractions: (a) the iron contained in the hemosiderin granules, (b) the iron in the soluble ferritin which is crystallizable with  $\text{CdSO}_4$ , designated simply as "ferritin," (c) the iron in a soluble but non-crystallizable substance (or substances) which is present in the mother liquor resulting from the crystallization of ferritin with  $\text{CdSO}_4$ . We shall refer preliminarily to this fraction as "non-crystallizable ferritin." The iron is in the ferric state, and it will be shown in a later paper dealing with magnetic measurements that the iron shows the same type of atomic structure in all of these fractions.

\* This is the first communication on closely related topics, all concerned with ferritin and some other ferric compounds, worked out by the collaboration of S. Granick, A. Rothen, and L. Michaelis, of The Rockefeller Institute for Medical Research, New York, and Charles D. Coryell of the Chemical Laboratory, University of California, Los Angeles.

### Method

*Isolation of Ferritin*—A few modifications were made in the method of isolation of ferritin as used by Laufberger and by Kuhn *et al.* Only one aqueous extract was made instead of two (2), since the yield on the second extraction was found to be negligible. After the aqueous spleen extract was heated to 80° (1), in order to expedite filtration, it was found convenient to remove the heavy precipitate by first running the hot suspension through cheese-cloth and then onto large fluted filters. (To increase the yield of filtrate, the precipitate in the cheese-cloth was pressed.) A clear red-brown solution resulted. (Since no precipitate was observed to form on the addition of 10 gm. of ammonium sulfate to 100 cc. of this red-brown solution, this step in the procedure of Kuhn *et al.* was omitted.) 30 gm. of ammonium sulfate were now added directly to each 100 cc. of the solution

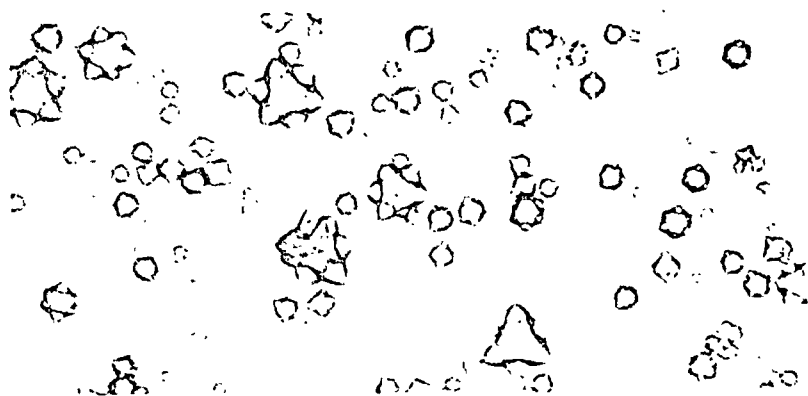


FIG. 1. Horse spleen ferritin;  $\times 250$

and the resulting suspension was kept in the ice box at 0° overnight. The precipitate was then centrifuged down and dissolved in distilled water. To crystallize out the ferritin, 4 to 5 gm. of cadmium sulfate ( $\text{CdSO}_4 \cdot 8/3\text{H}_2\text{O}$ ) were added per 100 cc. of this solution. Crystallization began within several minutes. After the solution had stood overnight, the crystals were separated off by centrifugation from a dark brown mother liquor that will no longer yield crystals. This solution will be referred to as "non-crystallizable ferritin." The crystals of ferritin (Fig. 1) which are sparingly soluble in distilled water we have found to be soluble in 2 per cent ammonium sulfate, yielding a clear deep red-brown solution. Ferritin in this solution can readily be crystallized by adding 4 to 5 gm. of cadmium sulfate per 100 cc. of solution.

The crystals of ferritin are not an artifact of the process of extracting and heating, since ferritin crystals can be seen to form on the under surface

of a cover-slip by treating a piece of teased horse spleen with a few drops of 10 per cent  $\text{CdSO}_4$  on a microscope slide.

A protein impurity, deep brown in color, may be removed from a dialyzed solution of crystallized ferritin by bringing it to  $\text{pH } 4.6 \pm 0.1$  with dilute acetate buffer (resulting ionic strength  $\mu = 0.1$ ) and letting it stand overnight. In some of the preparations, this fraction may be quite voluminous, so much so, that one might be misled in believing that the precipitate represents ferritin at its isoelectric point. However, in other samples this precipitate at  $\text{pH } 4.6$  may be very small. In any case, no crystalline ferritin can be obtained from this precipitate, although the supernatant fluid readily yields crystals with  $\text{CdSO}_4$ . Perhaps this material represents denatured protein. Preliminarily, one may classify it, together with "non-crystallizable ferritin," into a group of "non-crystallizable ferritins."

The recrystallized ferritin solutions, dissolved with the aid of ammonium sulfate, are dialyzed in cellophane tubes against slowly running distilled water until free from  $\text{NH}_4^+$  and  $\text{SO}_4^{=}$ .

*Analytical Methods*—The analytical methods used were checked on known mixtures of Fe, N, P, and Cd in the amounts to be expected in the unknowns, in the presence of added sucrose and edestin with appropriate blanks. For Fe and Cd determinations on the same sample, 25 to 50 mg. of ferritin were ashed wet with 1 cc. of concentrated  $\text{H}_2\text{SO}_4 + 0.2$  to 0.5 cc. of concentrated  $\text{HNO}_3$  in 100 cc. Kjeldahl flasks. The heating was continued gently at the stage of  $\text{SO}_3$  fumes for at least 30 minutes after clearing in order to oxidize the last traces of organic compounds. After cooling, 10 cc. of water were added and the solution was boiled for 10 minutes. To separate Fe from Cd, the solution was made 0.5 N with respect to  $\text{H}_2\text{SO}_4$  by neutralizing with strong NaOH solution (iron-free), adding the requisite amount of acid, and passing in  $\text{H}_2\text{S}$  (3). The precipitate of  $\text{CdS}$  was filtered through a modified Wintersteiner micro filter apparatus onto an asbestos mat contained in a 2 cc. capacity Gooch crucible. The filtrate was boiled to remove the  $\text{H}_2\text{S}$  and titrated with 0.04 N  $\text{KMnO}_4$ . The error in the determination of 4 to 10 mg. of Fe was  $\pm 0.5$  per cent. The  $\text{CdS}$  on the mat was brought into solution with a few cc. of hot 4 N  $\text{HCl}$  and filtered through another asbestos mat.

For amounts of Cd between 0.5 and 2.0 mg. in this filtrate, isolation as the 8-hydroxyquinoline complex was used. This reagent had been found satisfactory by Berg (4) for the macrodetermination of Cd. The procedure for the semimicrodetermination of Cd is described here in detail, since the conditions necessary for this determination are not evident from the literature (4). The hydrochloric acid solution containing  $\text{CdCl}_2$  was brought to  $\text{pH } 4$  to 5 (methyl red indicator) and diluted to a volume of 20 cc. 1 cc. of 8-hydroxyquinoline sulfate containing 10 mg. of the reagent was added;



then 4 M potassium acetate was added dropwise until a faint turbidity appeared, when 1 cc. more was added. The pH of this solution was 6 to 7. The solution was heated 10 minutes in a boiling water bath and the Cd complex was permitted to crystallize overnight and then filtered on a tared micro filter. It was dried at between 120–130° and weighed on the micro balance. The compound is  $\text{Cd}(\text{C}_6\text{H}_5\text{OH})_2$  (4). The error for 0.5 mg. of Cd sample was  $\pm 2$  per cent; for 1 to 2 mg. of Cd it was  $\pm 1$  per cent.

For amounts of Cd below 0.5 mg., a titrimetric dithizone procedure was used. This titrimetric method was suggested by Fischer (5) but the conditions for the quantitative determination of Cd by this means have not hitherto been described. The conditions to be described here were primarily worked out for the determination of Cd and iron in the same sample of ferritin. To separate small amounts of Cd from Fe, the CdS was left standing overnight before being filtered in order to form a coarser precipitate. The reagents were prepared for titration of solutions containing about 0.02 mg. of Cd per cc., although the procedure may be modified to determine as little as a few micrograms of Cd per cc. The dithizone solution was made up to contain 3 mg. of dithizone in 100 cc. of  $\text{CCl}_4$ . The reaction of Cd with dithizone was found to be quantitative between pH 6 and 7.5. In order to attain this region of pH readily, a solution of 4 M potassium acetate freed from metals was needed. This was prepared by shaking the 4 M potassium acetate in a separatory funnel for 5 minutes with 20 cc. of  $\text{CCl}_4$  containing 10 mg. of dithizone. The aqueous layer was then filtered through a moistened filter paper. A stop-cock grease insoluble in  $\text{CCl}_4$  was also prepared (6). The standardization of the dithizone solution is given as a typical example of the titrimetric dithizone procedure. To a 25 cc. separatory funnel were added 1 cc. of  $\text{CCl}_4$ , 1 cc. of a standard Cd solution containing 0.02 mg. of Cd in 0.01 N HCl, 2 cc. of  $\text{H}_2\text{O}$ , and 0.5 cc. of 4 M potassium acetate. The dithizone solution was now added from the burette in 0.5 cc. portions, and shaken for 15 seconds after each addition. The Cd reacted with the green  $\text{CCl}_4$  solution of dithizone to form an orange-pink Cd complex which was soluble in  $\text{CCl}_4$  but insoluble in water. When so much dithizone had been added that the  $\text{CCl}_4$  layer no longer turned orange-pink but remained green, the end-point had been exceeded. A slight orange scum formed in the funnel during the titration and this was removed as completely as possible by dissolving it in 3 to 5 cc. of  $\text{CCl}_4$ , shaking, and drawing off the  $\text{CCl}_4$  layer. With a little practice the titration end-point on two succeeding titrations could be determined within 0.05 cc. Since the amount of dithizone added was in the neighborhood of 3 cc., the error in the titration end-point was around 1.7 per cent.

Phosphorus was determined by ashing with sulfuric and nitric acids and by precipitation as ammonium phosphomolybdate, the precipitate being weighed on the micro balance (3). Phosphorus was also determined by the

colorimetric method of Youngburg and Youngburg (7). Nitrogen was determined by the Kjeldahl method with  $\text{CuSO}_4$  as catalyst and perhydrol to complete the digestion.

### *Physical Properties of Crystallized Ferritin*

*Changes on Heating Ferritin*—When a dialyzed ferritin solution containing 5 mg. of ferritin per cc. is heated slowly, at the rate of approximately 3–4° per minute, it remains clear at 80°. At 82° it becomes cloudy. When allowed to cool, the suspension becomes completely clear again in 20 minutes. If it is heated to 90°, an insoluble residue remains even on the next day, this residue constituting less than 25 per cent of the ferritin; the clear filtrate is readily crystallizable with  $\text{CdSO}_4$ . Still higher temperatures bring about a coagulation no longer reversible on cooling.

When dialyzed ferritin in a higher concentration (20 mg. per cc.) is heated, it becomes cloudy at 66° and when cool it clears completely within 5 minutes. Heating to higher temperatures delays the clearing. For example, if kept at 80–82° for 5 minutes, the ferritin becomes granular and requires 30 minutes to become clear, leaving only a trace of insoluble residue. If the hot solution containing the granular material is treated with an equal volume of 10 per cent  $\text{CdSO}_4$ , small irregular crystals are formed immediately. As the temperature decreases, well formed crystals begin to grow and the poorly formed ones disappear.

According to Chick and Martin (8), two phenomena are involved in heat coagulation of a protein. In modern terminology we may describe the one as a "denaturation," i.e. a chemical or structural change of the protein molecule itself, the second being an aggregation or "agglutination" of the individual protein molecules into particles larger than the wave-length of visible light. With many proteins, under certain conditions, especially of pH, denaturation by heat may occur before or without aggregation by heat. In the case of ferritin at higher concentrations (20 mg. per cc.), heat in the range between 60–80° causes the molecules to aggregate, this aggregation being reversible on cooling. Above 80°, changes in the individual molecules occur which are not reversible; such changed molecules agglutinate to insoluble granules. The special peculiarity of ferritin is that at sufficiently high concentrations the normal unchanged molecules can agglutinate before they denature. Others may interpret the facts by assuming two kinds of "denaturation," a reversible one occurring at 80° and an irreversible one occurring at higher temperatures. This depends on the definition of denaturation. It should be emphasized that the exact figure, 80°, depends on the conditions of these experiments; the temperature of denaturation, of course, varies with the concentration of protein and the rate of heating.

*Crystallization of Ferritin*—Crystals of ferritin form most readily when

the cadmium sulfate solution is added to a dialyzed ferritin solution. The rate of crystallization is high; for example, within 30 seconds after an equal volume of 10 per cent  $\text{CdSO}_4$  solution is added to a dialyzed aqueous ferritin solution (containing 2 per cent ferritin) one can observe well formed, growing crystals under the microscope. These crystals are optically isotropic, usually with slightly curved edges. The smaller ones are well formed octahedra; the large ones are twinned octahedra formed on the plan of a cube, on each face of which is set a tetrahedral pyramid. They are very soft, being easily crushed under a cover-slip; yet when crushed, they splinter into distinct fragments rather than smear out into a gel.

Crystals of the same shape may be obtained with  $\text{ZnSO}_4$  instead of  $\text{CdSO}_4$  but crystallization is more difficult. Laufberger reports obtaining crystals of ferritin even with cobalt and nickel sulfates. The crystallization of ferritin by means of Zn, Cd, Ni, or Co salts reminds one of the peculiarity of insulin which needs traces of any of these elements to form crystals. Ferritin, in contrast to insulin, requires over 3 per cent  $\text{CdSO}_4$  or  $\text{ZnSO}_4$  for crystallization.

When lower concentrations of  $\text{CdSO}_4$  are used for crystallization, several interesting phenomena are observed. Addition of  $\text{CdSO}_4$  to a ferritin solution so that the final solution contains 0.5 per cent  $\text{CdSO}_4$  (Experiment 1, Table I) results in the production of a precipitate which appears to be amorphous even under the oil immersion lens, and which remains so for weeks; results with a 0.7 per cent  $\text{CdSO}_4$  solution are similar (Experiment 2, Table I). At 1 per cent  $\text{CdSO}_4$ , flat plates are gradually formed. At 3 per cent  $\text{CdSO}_4$ , crystals of the typical octahedra and twinned crystals are formed rapidly and no amorphous precipitate is visible, the supernatant solution being colorless.

The amorphous precipitate formed at the lower concentrations of  $\text{CdSO}_4$  is not an impurity, for if 5 per cent  $\text{CdSO}_4$  is added directly to this amorphous precipitate (freed from its mother liquor) one may observe under the microscope that it dissolves and octahedral and twinned crystals arise rapidly.<sup>1</sup>

In Experiment 5 octahedral crystals of ferritin formed in 5 per cent  $\text{CdSO}_4$  were rapidly washed with 0.6 per cent  $\text{CdSO}_4$  and suspended in this solution. After several days, the original crystals had disappeared, giving way to small square plates which were optically anisotropic. This is the only case in which we have observed anisotropy in ferritin crystals. When these plates were brought into solution and 5 per cent  $\text{CdSO}_4$  was added, isotropic ferritin crystals were formed.

In order to find out whether these various amorphous precipitates and crystal forms represented substances of different cadmium content, the

<sup>1</sup> Dr. K. G. Stern had previously observed this phenomenon and mentioned it to us.

iron-cadmium ratios in the preparations were determined. Assuming an iron content in ferritin of 22 per cent, one can calculate from the Fe:Cd ratio the approximate percentage of Cd in the crystals. To remove the adhering mother liquor containing  $\text{CdSO}_4$  in the preparation of ferritin samples for analysis, the samples (Experiments 1 to 5) were centrifuged down, spread on porous porcelain to dry overnight, and then dried at  $110^\circ$ . The analyses (Table I) indicate that the amorphous precipitates (Experiment 1) contain not less than half the cadmium that the crystals contain (Experiment 4).

TABLE I  
*CdSO<sub>4</sub> Concentrations and Character of Ferritin Precipitates*

Ex- peri- ment No.	Composition	Character of ppts.	Ratio, Fe:Cd atoms	Per cent Cd in crystals
1	5 cc. Preparation VII (3 times crystallized ferritin), 0.5 cc. 0.4 M $\text{CdSO}_4$ , diluted to 10 cc. (0.5% $\text{CdSO}_4$ )	Amorphous ppt., optically isotropic	12.3:1	3.5
2	5 cc. Preparation VII, 0.7 cc. 0.4 M $\text{CdSO}_4$ , diluted to 10 cc. (0.7% $\text{CdSO}_4$ )	Amorphous ppt., somewhat greater than above, optically isotropic	10.0:1	4.4
3	5 cc. Preparation VII, 1.0 cc. 0.4 M $\text{CdSO}_4$ , diluted to 10 cc. (1% $\text{CdSO}_4$ )	Mostly flat, large plates or twinned, optically isotropic; slight amorphous ppt. (analyses made on plates)	8.5:1	5.2
4	5 cc. Preparation VII, 3.0 cc. 0.4 M $\text{CdSO}_4$ , diluted to 10 cc. (3% $\text{CdSO}_4$ )	Star-shaped twinned crystals and octahedra optically isotropic; no amorphous ppt.	6.6:1	6.7
5	Octahedra of Preparation VI (6 times crystallized), suspended in 0.6% $\text{CdSO}_4$	$15 \times 15 \mu$ , small square plates, optically anisotropic	9.2:1	4.8
6	Octahedra of Preparation VII, washed with saturated KCl	Crystals unchanged; optically isotropic	26:1	1.7

In Experiment 6, the adhering mother liquor was removed by rapidly washing ferritin crystals (originally precipitated with 5 per cent  $\text{CdSO}_4$ ) with saturated KCl in which these crystals were only slightly soluble. The Cd content decreased from 6.7 to 1.7 per cent. Since protein crystals appear to contain relatively large pores or capillary spaces (9), it appears that the  $\text{CdSO}_4$  present in aqueous solution in the pores of the crystal was swept out by the KCl solution without visibly affecting the crystalline form.

The  $\text{CdSO}_4$  may be considered to serve two functions: the first, to coordinate the molecules of ferritin into a definite lattice pattern; the second, to

decrease the solubility of ferritin, thus favoring crystallization. This latter function can be taken over by saturated KCl. Crystallization of ferritin could not be obtained, however, by using as the crystallizing solution 1 per cent  $\text{CdSO}_4$  in the presence of saturated KCl.

*Homogeneity of Ferritin*—In order to determine whether ferritin represented a pure protein, it was studied by the ultracentrifuge, electrophoretic, and solubility methods. The results of ultracentrifugation<sup>2</sup> indicated ferritin to be an inhomogeneous material consisting of large brown particles of molecular weight in the neighborhood of several million, grading down finally to a colorless fraction which appeared to make up 30 per cent of the protein. If these small colorless particles were considered to be spherical in shape, their molecular weight would be around half a million. Electrophoretic studies in the Tiselius apparatus,<sup>2</sup> however, indicated ferritin to be

TABLE II  
Solubility Studies on Ferritin

Tube No.	Ferritin in 0.4 per cent $\text{CdSO}_4 \cdot 8/3\text{H}_2\text{O}$		Ferritin in 0.6 per cent $\text{CdSO}_4 \cdot 8/3\text{H}_2\text{O}$	
	Ferritin		Ferritin	
	Suspension	Filtrate	Suspension	Filtrate
	mg. per cc.	mg. per cc.	mg. per cc.	mg. per cc.
1	0.260	0.256	0.678	0.591
2	1.44	0.990	1.18	1.05
3	2.10	1.34	2.79	2.06
4	3.92	1.85	5.32	2.45
5	4.39	1.88	6.50	2.50
6	5.77	1.97	10.2	2.64
7	8.19	2.21	13.0	2.73
8			17.9	3.02

a completely homogeneous substance with an isoelectric point below pH 5.4. The solubility method of Kunitz and Northrop (10) indicated ferritin to be inhomogeneous. This latter method was tested at two different concentrations of  $\text{CdSO}_4$ . Twice recrystallized ferritin was precipitated with 5 per cent  $\text{CdSO}_4$ ; the crystals were centrifuged down and washed twice by centrifugation with 0.4 per cent  $\text{CdSO}_4 \cdot 8/3\text{H}_2\text{O}$ . Increasing amounts of these washed crystals were then suspended in a series of tubes together with the same 0.4 per cent  $\text{CdSO}_4$  solution. After 24 hours of shaking at room temperature, which preliminary tests indicated as sufficient for the establishment of equilibrium, the tubes were centrifuged and aliquots of the

<sup>2</sup> Ultracentrifuge and electrophoretic studies on ferritin have been made by Dr. A. Rothen, who will report his data in another paper of this series.

brown supernatant solution were used for colorimetry in a Pulfrich photometer to determine the concentration of ferritin in solution. The results are given in Table II. In another experiment 0.6 per cent  $\text{CdSO}_4 \cdot 8/3\text{H}_2\text{O}$  was used as solvent with a ferritin preparation that had been recrystallized six times; the results are also reported in Table II.

### *Some Chemical Properties of Ferritin*

*Iron Content*—Further evidence for the inhomogeneity of ferritin is the variability of the iron, phosphorous, and nitrogen content of different crystallized ferritin samples. Iron analyses of these ferritin samples after

TABLE III  
*Analyses of Non-Hemin Iron Components of Horse Spleen in Per Cent of Dry Weight\**

Preparation	Fe		Cd	P	N
	Uncorrected for Cd	Corrected for Cd	Uncorrected for Cd		
	per cent	per cent	per cent	per cent	per cent
I. Ferritin crystallized twice..	22.2	22.7	2.88	1.60	10.55
II. " " 3 times; removal of Cd by ammoni- um sulfate and dialysis...	22.7	22.8	0.724	1.26	
IX. Ferritin crystallized 4 times.	19.7	20.2	2.74	1.42	11.1
X. " " once; from a horse severely bled several mos.....	22.6	23.1	1.93	1.99	12.6
IV. Fraction pptd. with $\text{Na}_2\text{SO}_4$ (not crystallized).....	24.5			1.45	11.0
VIII. Hemosiderin granules.....	8.29			1.59	12.9
VII. "Non-crystallizable ferritin" from mother liquor.....	19.8	20.2	2.02	1.52	9.12

\* All preparations were dried in thin layers at  $80^\circ$  for 24 hours and then for 3 hours at  $110^\circ$ .

dialysis vary from 20.2 to 23.1 per cent if the Cd adhering to the ferritin is considered as an impurity and is corrected for.

It was interesting to see what the iron content of a preparation would be if it were not crystallized with  $\text{CdSO}_4$ , but merely fractionated with  $\text{Na}_2\text{SO}_4$ . Preparation IV (Table III) was therefore made in the following manner. The filtrate of the horse spleen extract after being heated to  $80^\circ$  was precipitated with ammonium sulfate in the usual manner. This precipitate was then dissolved in water to give a deep brown, clear solution which was fractionated into three parts by  $\text{Na}_2\text{SO}_4$  of increasing concentration up to saturation. There is no particularly sharp separation with this procedure, since the precipitates form in a concentration of  $\text{Na}_2\text{SO}_4$  near saturation.

The intermediate fraction was arbitrarily chosen and was dialyzed free from salts. This Preparation IV had an iron content of 24.5 per cent.

The brown mother liquor from a  $\text{CdSO}_4$  precipitation (designated as "non-crystallizable ferritin") was precipitated with ammonium sulfate, dialyzed, and centrifuged. The supernatant liquid soluble in the absence of ammonium sulfate yields a preparation, No. VII, having 19.8 per cent iron and somewhat less nitrogen than the crystallized ferritin preparation.

Hemosiderin granules isolated by differential centrifugation in a partial state of purity had an iron content of 8.29 per cent, a nitrogen content of 12.9 per cent, and a phosphorus content of 1.6 per cent. It is not possible to say whether this phosphorus is a component of the granules or not.

*Phosphorus Content*—The phosphorus content of crystallized ferritin preparations was also variable, ranging from 1.26 to 2.00 per cent. Kuhn and his coworkers have postulated this phosphorus to be a constituent of a desoxyribonucleic acid. We have been unable to confirm the presence of any nucleic acid in any of our crystallized samples of ferritin. When ferritin was made 1 N with NaOH, within a few minutes at room temperature a dark brown, flocculent precipitate formed. This precipitate, Fraction A, contained all of the iron, 5 per cent of the nitrogen, and 23 per cent of the phosphorus. The supernatant liquid, Fraction B, was colorless and contained 77 per cent of the phosphorus in the form of inorganic orthophosphate. Fraction B also contained 85 per cent of the total N in the form of a substance precipitated at pH 4.6 and identified as a protein by the biuret, ninhydrin, and Millon tests. Both Fractions A and B were tested for pentoses with Bial's reagent, for desoxypentose by the Kiliani method, and with the Dische diphenylamine reagent; all tests were negative. To detect purines, absorption spectra in the ultraviolet region were taken by Dr. G. Lavin on both fractions, and again with negative results. It is not yet possible to say whether the phosphate is in some very labile organic combination or whether the phosphate, perhaps as a basic ferric phosphate, is a part of a colloidal micelle of ferric hydroxide. Consequently, if some preparations of ferritin, such as Kuhn's, should contain nucleic acid, this should be considered as a non-essential admixture.

*Cadmium Content*—It has not been found possible, by dialysis against distilled water, to remove all the cadmium from a solution prepared from ferritin-cadmium crystals. Prolonged dialysis until the dialysate is free from sulfate ions gives a preparation containing from 2 to 3 per cent cadmium (Preparations I, IX, X, Table III). We have been able to decrease the cadmium content to 0.72 per cent by washing the ferritin-cadmium with ammonium sulfate (Preparation II) in the following manner. A thrice crystallized ferritin solution was precipitated with 30 per cent ammonium sulfate, the flocculent precipitate was washed with a fresh solution of am-

monium sulfate, the ferritin was redissolved, and the precipitation and washing repeated. Finally the precipitate was dissolved and dialyzed until free from ammonium ions and its cadmium content determined. Ferritin seems to hold cadmium as tenaciously as, according to Schorn (11), egg albumin treated with zinc ions holds the zinc, of which 0.63 per cent still remains even on electrodialysis.

#### SUMMARY

Horse spleen ferritin, a protein containing over 20 per cent iron, and crystallizable as a cadmium salt is not an artifact of the methods of isolation and heating, since crystals can be obtained directly by treating a piece of teased horse spleen with  $\text{CdSO}_4$ . Ferritin is homogeneous according to the Tiselius electrophoretic method. It is, however, inhomogeneous in the ultracentrifuge, being made up of brown particles having a molecular weight in the neighborhood of several million and grading down to colorless particles of the size of large globulin molecules. Solubility studies also give evidence for the inhomogeneity of crystallized ferritin. Inhomogeneity is further indicated by the variation in the iron and phosphorus content of different ferritin samples. No evidence was obtained for the presence of nucleic acid in ferritin. The effect of heat on ferritin is peculiar, since a 3 per cent solution of ferritin when heated to  $60^\circ$  gives a coagulum which redissolves on cooling; when heated to higher temperatures, a coagulum is produced having the appearance of an irreversibly denatured protein. Peculiarities of crystallization and the cadmium content of the crystals are discussed. Methods for cadmium analyses by means of 8-hydroxyquinoline and by a titrimetric dithizone procedure are described.

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# SYNTHESIS OF PEPTIDES OF *l*-SERINE

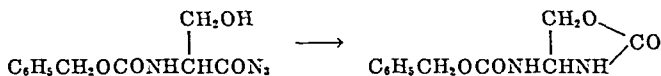
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Recently, a method has been developed (1) for the isolation of *l*-serine from silk fibroin. The ready availability of this amino acid has now made it possible to apply the carbobenzoxy method (2) to the synthesis of peptides containing *l*-serine. In the present communication, syntheses of *l*-serylglycine, *l*-seryl-*l*-alanine, *l*-seryl-*l*-serine, and *l*-seryl-*l*-glutamic acid are described. The syntheses involve the reaction of carbobenzoxy-*l*-serinazide with the appropriate amino acid ester. Saponification of the coupling products followed by catalytic hydrogenation yields the expected peptides.

When an ethyl acetate solution of carbobenzoxy-*l*-serinazide is heated to 40° for 20 minutes, 4-carbobenzoxyaminooxazolidone-2 is formed.<sup>1</sup>



In coupling carbobenzoxy-*l*-serinazide with amino acid esters, it is advisable therefore to keep the temperature of the reaction below 25° in order to prevent appreciable formation of the oxazolidone.

A preparation of *l*-seryl-*l*-serine has been described previously by Fischer (4). This material was prepared by partial hydrolysis of *l*-serine anhydride obtained from silk fibroin. Fischer questioned the optical purity of his material which had a specific rotation in normal hydrochloric acid of +12.0°. The rotation found for *l*-seryl-*l*-serine synthesized by the carbobenzoxy method was +14.2°.

The synthesis of peptides of *l*-phosphoserine by phosphorylation of the corresponding carbobenzoxyserine peptide esters is contemplated. The phosphorylated *l*-seryl-*l*-glutamic acid is of special interest since Posternak and Pollaczek (5) have presented evidence to show that the peptide obtained by Levene and Hill (6) from casein is phosphoserylglutamic acid. The former workers have also reported that this phosphopeptide is resistant to dipeptidase action; however, removal of the phosphoric acid residue renders the peptide bond sensitive to enzyme action.

At the outset of this investigation it was intended to examine the speci-

<sup>1</sup> Similar ring formation was observed by Schroeter (3) on heating  $\beta$ -phenylhydracrylic acid azide.

city of the action of proteolytic enzymes on serine peptides. However, owing to present circumstances, the enzymatic studies could not be completed.

TABLE I

*Hydrolysis of Dipeptides Containing l-Serine by Swine Intestinal Mucosa*

Substrate concentration, 0.05 mm per cc. of test solution; enzyme concentration, 0.25 mg. of protein N per cc. of test solution; temperature 40°.

Substrate	pH	Time	Hydrolysis
		hrs.	per cent
<i>l</i> -Serylglycine	7.5	23	23
		47	56
<i>l</i> -Seryl- <i>l</i> -alanine	7.7	23	29
		47	59
<i>l</i> -Seryl- <i>l</i> -serine	7.5	23	18
		47	35
Glycylglycine	7.8	23	29
		47	54
<i>l</i> -Leucylglycine	7.8	3	16
		6.5	32
<i>l</i> -Leucylglycylglycine	7.7	1	37
		3	86

TABLE II

*Hydrolysis of Carbobenzoxy-l-serinamide by Intracellular Proteinases*

Substrate concentration, 0.05 mm per cc. of test solution; concentration of activator (cysteine), 0.01 mm per cc. of test solution; temperature 40°; pH 4.9.

Enzyme	Protein N per cc. test solution	Time	Hydrolysis	K*
	mg.	min.	per cent	
Cysteine-papain	0.276	80	40	0.0028
		170	65	0.0027
		240	76	0.0026
Cysteine-beef spleen cathepsin	0.346	80	8	0.00045
		170	19	0.00054
		240	23	0.00047

$$* K = \frac{1}{\text{min.}} \log \frac{100}{100 - \% \text{ hydrolysis}}$$

Preliminary experiments showed that several of the dipeptides described in the present communication are split by an aqueous extract of swine intestinal mucosa. It will be noted from Table I that the rate of enzymatic hydrolysis of the serine dipeptides is rather similar to the rate of splitting of glycylglycine. On the other hand, *l*-leucylglycine and *l*-leucylglycylglycine are hydrolyzed much more rapidly.

Carbobenzoxy- $\beta$ -serinamide is split by cysteine-papain and by cysteine-beef spleen cathepsin (Table II). The hydrolyses follow the kinetics of a first order reaction. This enzymatic action may be attributed to a proteinase (endopeptidase) in each of the two enzyme preparations. It remains to be determined whether the hydrolysis of carbobenzoxyserinamide is due to the action of any of the hitherto recognized proteinases in papain or beef spleen cathepsin preparations (7).

The author wishes to express his thanks to Dr. Max Bergmann for kind advice and encouragement and to Miss Rosalind Joseph and Mr. Stephen M. Nagy for valuable assistance in this investigation.

#### EXPERIMENTAL

*Carbobenzoxy-l-serine*—3.2 gm. of *l*-serine ( $[\alpha]_D^{27} = +14.8^\circ$  (10 per cent in 2 N HCl) (1)) were dissolved in 30 cc. of N NaOH. 5 gm. of carbobenzoxy chloride and 10 cc. of 4 N NaOH were added in three portions with cooling and shaking. Concentrated hydrochloric acid was added carefully to Congo red acidity. The syrup which appeared crystallized quickly. The crude product was dried on a porous plate and recrystallized from hot chloroform. Yield, 4.5 gm.; m.p.,  $121^\circ$ .

$C_{11}H_{13}O_4N$ .	Calculated.	C 55.2, H 5.5, N 5.9
239.2	Found.	" 55.0, " 5.8, " 5.8
$[\alpha]_D^{25} = +5.6^\circ$	(6% in glacial acetic acid)	

*Carbobenzoxy-l-serinhydrazide*—3.2 gm. of *l*-serine were esterified with methanol and dry HCl. The syrupy ester was coupled with 5 gm. of carbobenzoxy chloride (in two portions). Potassium bicarbonate was added to neutralize the HCl and ethyl acetate was used as the organic solvent. The excess carbobenzoxy chloride was destroyed with pyridine and the ethyl acetate layer was washed successively with water, hydrochloric acid, and water and finally dried over  $Na_2SO_4$ . On evaporation, a syrup resulted which was dissolved in 50 cc. of absolute alcohol and 1.5 cc. of hydrazine hydrate were added. Crystallization of the hydrazide was complete after 24 hours at room temperature. Yield, 3.4 gm.; m.p.,  $181^\circ$ .

$C_{11}H_{13}O_4N_2$ .	Calculated.	C 52.2, H 6.0, N 16.6
253.2	Found.	" 52.2, " 6.0, " 16.5

*Carbobenzoxy-l-serinamide*—The syrupy carbobenzoxy-*l*-serine methyl ester (from 5 gm. of *l*-serine) was dissolved in 100 cc. of methanol previously saturated with dry ammonia at  $0^\circ$  and allowed to stand at room temperature for 2 days. On evaporation of the solvent, crystals were obtained.

The material was recrystallized from ethyl acetate. Yield, 4.7 gm.; m.p., 132–133°.

$C_{11}H_{14}O_4N_2$ .	Calculated.	C 55.5, H 5.9, N 11.8
238.2	Found.	" 55.5, " 6.0, " 11.8
$[\alpha]_D^{25} = +14.4^\circ$ (5% in ethanol)		

*l*-Serylglycine

*Carbobenzoxy-l-serylglycine Benzyl Ester*—3.5 gm. of carbobenzoxy-*l*-serinhydrazide were suspended in 40 cc. of water and 3 cc. of glacial acetic acid and 1 cc. of concentrated hydrochloric acid were added to give a clear solution. With cooling and shaking, a solution of 1.5 gm. of sodium nitrite in 10 cc. of water was added slowly. The resulting syrupy azide was extracted with ethyl acetate. The ethyl acetate layer was quickly washed with cold water, bicarbonate, and water and then dried over  $Na_2SO_4$ . The dry ethyl acetate solution was added to an ethereal solution of glycine benzyl ester (prepared from 8 gm. of the hydrochloride). After 20 hours at room temperature, the reaction mixture was washed with dilute hydrochloric acid, water, bicarbonate, and water and dried over  $Na_2SO_4$ . On evaporation to a small volume and addition of petroleum ether, crystals were obtained. Yield, 2.4 gm.; m.p., 102°.

$C_{20}H_{22}O_6N_2$ .	Calculated.	C 62.2, H 5.8, N 7.3
386.4	Found.	" 62.1, " 5.8, " 7.4

*l*-Serylglycine—1.0 gm. of carbobenzoxyserylglycine benzyl ester was hydrogenated in methanol in the presence of palladium black. The peptide separated out during the hydrogenation and was dissolved by addition of water. The filtrate from the catalyst was evaporated. On addition of alcohol, crystallization of the peptide occurred. Yield, 0.4 gm.

$C_3H_{10}O_4N_2$ .	Calculated.	C 36.8, H 6.2, N 17.3
162.1	Found.	" 36.8, " 6.4, " 17.1
$[\alpha]_D^{25} = +30.2^\circ$ (6% in $N$ HCl)		

This peptide was also prepared by hydrogenation of carbobenzoxy-*l*-serylglycine (described below).

*Carbobenzoxy-l-serylglycine Ethyl Ester*—An ethyl acetate solution of carbobenzoxy-*l*-serinazide (prepared as above from 1 gm. of the hydrazide) was added to an ethereal solution of glycine ethyl ester (from 5 gm. of the hydrochloride). After 24 hours at room temperature, the reaction mixture was washed with dilute hydrochloric acid, water, bicarbonate, and water and finally dried over  $Na_2SO_4$ . On concentration of the solution to a small volume and addition of petroleum ether, crystals were obtained. Yield, 0.8 gm.; m.p., 105–107°.

$C_{15}H_{20}O_6N_2$ .	Calculated.	C 55.5, H 6.2, N 8.6
324.3	Found.	" 55.5, " 6.2, " 8.5

*Carbobenzoxy-l-serylglycine*—1 gm. of the ethyl ester was dissolved in 25 cc. of methanol and 3.4 cc. of N NaOH were added. After 30 minutes at room temperature, 3.8 cc. of N HCl were added and the methanol was evaporated off. The aqueous solution was placed in the ice box. After 3 days the material had crystallized out. Yield, 0.5 gm. The melting point after recrystallization from hot water was 131°.

$C_{12}H_{16}O_6N_2$ .	Calculated.	C 52.7, H 5.4, N 9.5
296.2	Found.	" 52.5, " 5.3, " 9.6

### *l-Seryl-l-alanine*

*Carbobenzoxy-l-seryl-l-alanine Methyl Ester*—10 gm. of carbobenzoxy-*l*-serinhydrazide were converted to the azide in the usual manner. The ethyl acetate solution of the azide was added to a solution of *l*-alanine methyl ester (prepared from 20 gm. of the syrupy hydrochloride), concentrated to one-third of the original volume, and left to stand overnight. After the solution was washed with dilute hydrochloric acid, water, bicarbonate, and water, the non-aqueous layer was dried and concentrated under reduced pressure. Addition of petroleum ether precipitated the material in crystalline form. Yield, 7.8 gm. After recrystallization from ethyl acetate-ether, the substance melted at 113–114°.

$C_{15}H_{20}O_6N_2$ .	Calculated.	C 55.5, H 6.2, N 8.6
324.3	Found.	" 55.6, " 6.3, " 8.7

*Carbobenzoxy-l-seryl-l-alanine*—3.2 gm. of the methyl ester were dissolved in 30 cc. of methanol and 11 cc. of N NaOH were added. After 30 minutes, 12 cc. of N HCl were added and the methanol was evaporated off. The residue was chilled and the crystals collected. Yield, 2.5 gm. The substance was recrystallized from hot water.

$C_{14}H_{18}O_6N_2 \cdot \frac{1}{2}H_2O$ .	Calculated.	C 52.7, H 6.0, N 8.8, H <sub>2</sub> O 2.8
319.3	Found.	" 52.7, " 6.1, " 8.7, " 2.7

After drying at 100° for 3 hours *in vacuo* over P<sub>2</sub>O<sub>5</sub>, the substance melted at 161–162°.

*l-Seryl-l-alanine*—1 gm. of the carbobenzoxy compound was hydrogenated in methanol in the presence of palladium black. The peptide separated out during the hydrogenation and was dissolved by the addition of hot water. The filtrate from the catalyst was evaporated down to a small volume. The peptide was crystallized by the addition of absolute alcohol. Yield, 0.5 gm.

$C_6H_{12}O_4N_2$ .	Calculated.	C 40.9, H 6.9, N 15.9
176.2	Found.	" 40.7, " 7.0, " 15.7
[ $\alpha$ ] <sub>D</sub> <sup>25</sup> = -30.4° (6% in N HCl)		

*l*-Seryl-*l*-serine

*Carbobenzoxy-l-seryl-l-serine Methyl Ester*—An ethyl acetate solution of carbobenzoxyserinazide (prepared from 3.5 gm. of the hydrazide) was added to an ethyl acetate solution of serine methyl ester (from 5 gm. of hydrochloride). After 20 hours at room temperature crystals (0.6 gm.) had separated out. The filtrate was washed with dilute HCl, water, bicarbonate, and water. After drying over Na<sub>2</sub>SO<sub>4</sub>, the solution was concentrated to a small volume and petroleum ether was added. A second crop of crystals was obtained. The total yield was 1.4 gm.; m.p., 143–145°.

C <sub>15</sub> H <sub>20</sub> O <sub>7</sub> N <sub>2</sub> .	Calculated.	C 52.9, H 5.9, N 8.2
340.4	Found.	" 52.9, " 6.1, " 8.3

*Carbobenzoxy-l-seryl-l-serine*—1.0 gm. of the methyl ester was dissolved in 15 cc. of methanol and 3.2 cc. of *N* NaOH were added. The reaction mixture was left at room temperature for 20 minutes and then acidified with 3.6 cc. of *N* HCl. On evaporation of the methanol under reduced pressure and chilling overnight, 0.5 gm. of crystals was obtained. After recrystallization from hot water the substance melted at 169–171°.

C <sub>14</sub> H <sub>18</sub> O <sub>7</sub> N <sub>2</sub> .	Calculated.	C 51.5, H 5.6, N 8.6
326.3	Found.	" 51.4, " 5.6, " 8.6

*l*-Seryl-*l*-serine—1.1 gm. of the carbobenzoxy compound were hydrogenated catalytically in methanol solution. The catalyst was filtered off and washed with hot water. The filtrate and washings were combined, concentrated to a small volume, and chilled. The crystalline peptide was filtered off and dried *in vacuo* over P<sub>2</sub>O<sub>5</sub>. Yield, 0.5 gm. The material is sparingly soluble in cold water.

C <sub>8</sub> H <sub>12</sub> O <sub>5</sub> N <sub>2</sub> .	Calculated.	C 37.5, H 6.3, N 14.6
192.2	Found.	" 37.3, " 6.4, " 14.4
[α] <sub>D</sub> <sup>20</sup> = +14.2° (7% in <i>N</i> HCl)		

*l*-Seryl-*l*-glutamic Acid

*Carbobenzoxy-l-seryl-l-glutamic Acid Diethyl Ester*—An ethyl acetate solution of carbobenzoxy-*l*-serinazide (prepared from 2.8 gm. of the hydrazide) was added to an ethyl acetate solution of glutamic acid diethyl ester (prepared from 10 gm. of the hydrochloride). The reaction mixture was allowed to stand at 20° overnight, washed with dilute hydrochloric acid, water, dilute bicarbonate, and water. After drying over Na<sub>2</sub>SO<sub>4</sub>, the solution was concentrated under reduced pressure. The resulting syrup was crystallized by the addition of petroleum ether. Yield, 2.9 gm. After recrystallization from ether-petroleum ether, the substance melted at 85–86°.

C <sub>20</sub> H <sub>28</sub> O <sub>8</sub> N <sub>2</sub> .	Calculated.	C 56.6, H 6.6, N 6.6
424.4	Found.	" 56.5, " 6.5, " 6.5

*Carbobenzoxy-l-seryl-l-glutamic Acid*—1.3 gm. of the diethyl ester were dissolved in 20 cc. of methanol and 6.3 cc. of  $N$  NaOH were added. After 30 minutes, 6.7 cc. of  $N$  HCl were added and the reaction mixture concentrated under reduced pressure. The resulting oil crystallized on standing in the ice box. Yield, 0.8 gm. After recrystallization from hot water, the substance melted at 152–153°.

$C_{15}H_{20}O_5N_2$ .	Calculated.	C 52.2, H 5.5, N 7.6
368.4	Found.	" 52.0, " 5.6, " 7.7

*l-Seryl-l-glutamic Acid*—0.5 gm. of the carbobenzoxy compound was hydrogenated in methanol in the presence of palladium black. The peptide separated out during the hydrogenation and was dissolved by the addition of hot water. The filtrate from the catalyst was evaporated down to a small volume. The peptide was crystallized by the addition of absolute alcohol. Yield, 0.3 gm.

$C_8H_{14}O_6N_2$ .	Calculated.	C 41.0, H 6.0, N 12.0
234.2	Found.	" 41.0, " 6.0, " 11.8
$[\alpha]_D^{25} = -9.4^\circ$ (6% in $N$ HCl)		

*4-Carbobenzoxyaminooxazolidone-2*—An ethyl acetate solution of carbobenzoxy-*l*-serinazide (prepared in the usual manner from 2 gm. of the hydrazide) was heated to 40° for 20 minutes. Addition of petroleum ether yielded a crystalline precipitate. Yield, 1.2 gm. After recrystallization from hot water, the substance melted at 171°.

$C_{11}H_{12}O_4N_2$ .	Calculated.	C 55.9, H 5.1, N 11.9
236.2	Found.	" 55.9, " 5.1, " 11.9

75 mg. of this substance were refluxed with 10 per cent hydrochloric acid for 2 minutes. On cooling, crystals (24 mg.) separated out. M.p., 90–91°. The product was benzyl carbamate (8, 9).

$C_8H_9O_2N$ .	Calculated.	C 63.7, H 6.0, N 9.3
151.2	Found.	" 64.0, " 6.1, " 9.2

An authentic sample of benzyl carbamate was prepared by treatment of carbobenzoxy chloride with ammonia in ether. A mixed melting point with the product obtained by treatment of the oxazolidone with hydrochloric acid showed no depression.

### *Enzymatic Studies*

The preparation of swine intestinal mucosa was a crude aqueous extract kindly supplied by Dr. E. L. Smith. The papain (10) and the beef spleen cathepsin solution (11) were prepared as described previously. The substrate concentration was 0.05 mM per cc. of test solution. 0.1 M



veronal buffer was employed for the experiments at pH 7.5 to 7.8; 0.2 M citrate buffer was used for the experiments at pH 4.9. The extent of hydrolysis was followed by titration of liberated carboxyl groups by the method of Grassmann and Heyde (12).

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# THE ELECTROPHORETIC MOBILITIES OF DESOXYRIBOSE AND RIBOSE NUCLEIC ACIDS

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Electrophoresis of desoxyribose nucleic acid (DNA) (1, 2) and ribose nucleic acid (RNA) (3, 4) has indicated in the main that the former possesses a higher mobility than the latter. Similarly, titration data have indicated that, assuming a tetranucleotide unit, DNA possesses five acidic groups per tetranucleotide, while RNA has four (5, 6). Treatment with ribonuclease has been found to increase the acidic charge of RNA from four to five (7). However, titration (8, 9) as well as electrophoretic (10) data have also been presented to show that DNA and RNA possess the same net charge. Hence, the comparison in a number of laboratories of the electrophoretic mobilities of different nucleic acids has led to discordant results. However, the procedures employed have not been sufficiently uniform with respect to ionic strength, pH, electrolyte, etc., to permit a rigorous comparison of the data. Therefore, in the present work artificial mixtures of various desoxyribose nucleic acids and ribose nucleic acids were studied by the electrophoretic method in an attempt to clarify the reported discrepancies.

## EXPERIMENTAL

Yeast RNA (Merck) was purified by the method of Kunitz (11). The author is indebted to Dr. M. Kunitz of the Rockefeller Institute for samples of thymus DNA prepared according to Levene and of crystalline ribonuclease, and to Dr. K. Meyer of Columbia University for a sample of thymus DNA prepared according to Hammarsten. The Levene DNA was far less viscous in solution than that prepared by Hammarsten's method.

The Levene DNA and the mixtures listed in Table I were examined by the Longworth technique in the Tiselius electrophoresis apparatus at pH 7.0 in 0.02 *N* veronal buffer containing 0.08 *N* sodium chloride. The concentration of each type of nucleic acid was 0.2 per cent. An aliquot of the Levene DNA and RNA mixture incubated with 0.03 per cent ribonuclease at 30° for 24 hours was examined without reequilibration against the buffer.

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The pH and conductivities were practically unchanged. The mobilities of the separate components after migrating about 3 cm. are given in Table I. The mobilities of the descending boundaries are used in subsequent comparisons.

TABLE I  
*Electrophoretic Mobilities of Components in Nucleic Acid Mixtures*

Nucleic acid mixtures	Boundary mobilities $\times 10^3$ cm. <sup>2</sup> sec. <sup>-1</sup> volt <sup>-1</sup>	
	Ascending	Descending
Levene DNA .....	-14.2	-14.0
" " + RNA .....	-15.0	-14.3
" " + ribonuclease-treated RNA ..	-14.8, -18.3	-13.9, -16.8
RNA + Hammarsten DNA .....	-14.8, -17.5	-13.6, -16.4

### Results

The DNA and the admixed RNA, both prepared according to Levene, migrated as one component. The former possessed essentially the same mobility alone as in the mixture. The DNA prepared according to Hammarsten migrated with a very sharp boundary whose mobility was 21 per cent greater than that of the admixed RNA. Ribonuclease-treated RNA yielded a new boundary whose mobility was 21 per cent greater than that of the original RNA or of admixed DNA prepared according to Levene. Therefore, the Hammarsten DNA possessed essentially the same mobility as ribonuclease-treated RNA. When a mixture of these last two substances was examined electrophoretically, it migrated in the main as a sharp spike with a rapidly spreading base.

The 21 per cent higher mobility of the Hammarsten DNA and ribonuclease-treated RNA, as compared to DNA and RNA isolated according to Levene, is considered to be in good agreement with the five acidic groups assigned to the former substances and the four groups ascribed to the latter preparations. The reason for the decreased acidity of the alkaline-treated Levene DNA is not clear. The fifth acidic group, whatever its nature, may be responsible for the considerable particle interaction of the Hammarsten DNA.

The finding, by means of titration, that ribonuclease liberates acidic groups in RNA has been confirmed by means of the electrophoretic technique. These groups are not necessarily similar to those of the Hammarsten DNA. In this case, however, it is probable that secondary phosphoric acid groups have been liberated and, here at least, do not result in increased particle interaction.

Since it appears that both RNA and DNA may possess either four or five

acidic groups per assumed tetranucleotide, depending upon the previous treatment, it is not surprising that the data in the literature seem somewhat inconsistent. It is obvious from the present results that, in the characterization of different nucleic acids, the treatment in the course of isolation, such as the degree of tissue autolysis, reagents used, etc., as well as the chemical and physical properties, should be described.

#### SUMMARY

Various nucleic acids have been studied electrophoretically. The effect of preparative treatment on electrophoretic mobility, and hence on charge, has been demonstrated and discussed. Desoxyribose nucleic acid and ribose nucleic acid may possess similar or different charges, depending on the method of isolation.

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# THE STRUCTURE OF BIOTIN: A STUDY OF DESTHIOBIOTIN

BY VINCENT DU VIGNEAUD AND DONALD B. MELVILLE\*

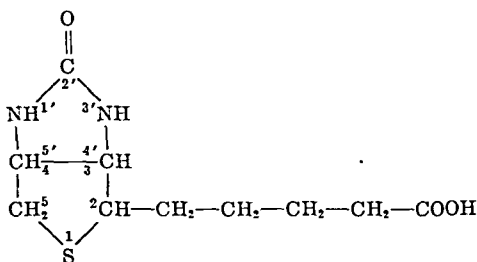
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JOHN C. KERESZTESY, AND STANTON A. HARRIS

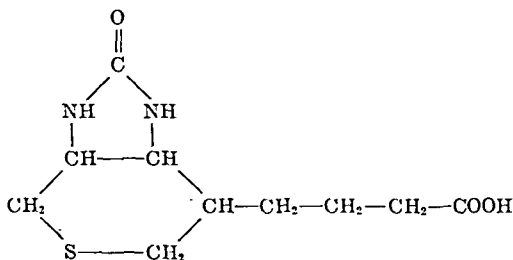
(From the Research Laboratory of Merck and Company, Inc., Rahway, New Jersey)

(Received for publication, September 25, 1942)

Structural studies on biotin in the previous paper (1) showed that the molecule probably possessed either structure (I) or (II). Structure (I) appeared to be more acceptable than (II) for the interpretation of experimental data, particularly the formation of adipic acid from the oxidation



(I)



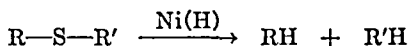
(II)

(2) of the diaminocarboxylic acid derived from biotin (3). However, since the oxidative mechanism of the formation of adipic acid might have involved the decarboxylation of an intermediary malonic or  $\beta$ -keto acid

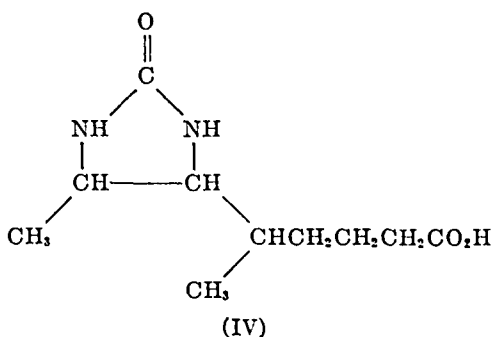
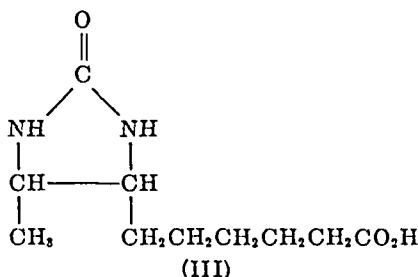
\* Acknowledgment is made to the S. M. A. Corporation in appreciation of a research grant which has made part of this work possible.

derivative from (II), this structure was not eliminated. Evidence from additional degradation reactions described herein eliminates structure (II) from consideration and establishes the preferred formulation (I) as the structure of biotin.

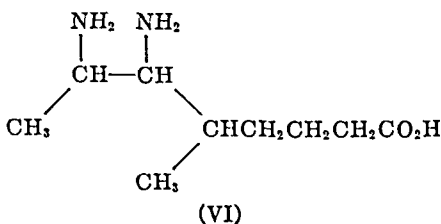
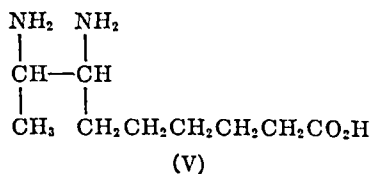
It was believed that organic sulfides could be cleaved by the Raney nickel catalyst in the absence of a hydrogen atmosphere according to the equation



If this reaction were successful on biotin, the desthiobiotin produced would have either structure (III) or (IV) as based upon structures (I) and (II) respectively for biotin. Just as the ureido ring of biotin is hydrolyzed (3) to the diaminocarboxylic acid, the ureide (III) should yield  $\zeta, \eta$ -diamino-

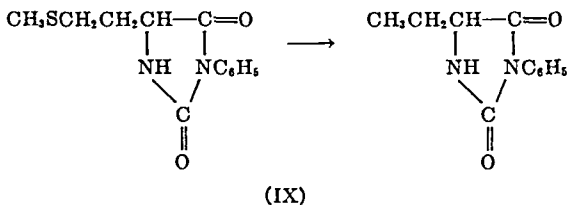
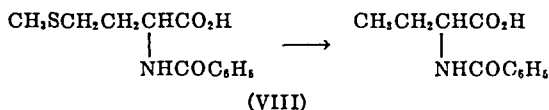
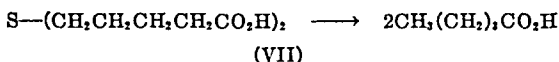


pelargonic acid (V) on hydrolysis, and the ureide (IV) should yield  $\delta$ -methyl- $\epsilon, \zeta$ -diaminocaprylic acid (VI). The diamino acids (V) and (VI) contain one and two carbon-methyl groups respectively. Thus, a Kuhn-



Roth carbon-methyl group determination on the desthiiodiaminocarboxylic acid so obtained from biotin would quickly differentiate between structures (V) and (VI). More positive characterization of the desthiiodiaminocarboxylic acid could be established by an oxidative cleavage reaction. Pimelic acid would be formed, if its structure were (V), and  $\alpha$ -methyladipic acid would be formed if its structure were (VI).

Because of the limited amounts of available crystalline biotin, the sulfide cleavage reaction over Raney's nickel was tried on "model" compounds. The sulfides (VII), (VIII), and (IX), which possess certain structural features of biotin, were cleaved to their corresponding sulfur-free products in yields of 65 to 95 per cent on both a macro and semimicro scale. These reactions and the results of subsequent studies on sulfur compounds not related to biotin are described in detail elsewhere.<sup>1</sup> When biotin methyl



ester was treated similarly, a product containing the same number of carbon atoms and 2 added hydrogen atoms and no sulfur atom was obtained. Whereas earlier studies had strongly indicated the cyclic nature of the sulfide group in biotin, the formation of desthiobiotin provided definite proof of it.

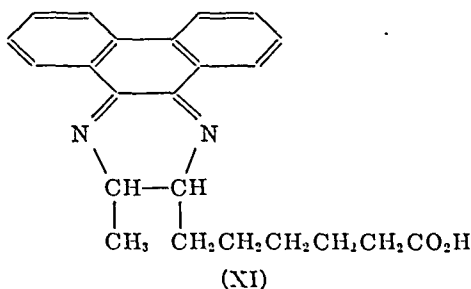
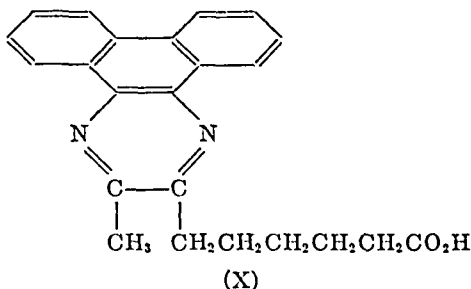
Desthiobiotin methyl ester was hydrolyzed under several conditions by aqueous HCl (4), and the corresponding desthiiodiaminocarboxylic acid was isolated as the dihydrochloride. The Ba(OH)<sub>2</sub> method (3) of hydrolysis yielded the desthiiodiaminocarboxylic acid sulfate, and this salt was obtained more satisfactorily than the dihydrochloride. A carbon-methyl group determination on the desthiiodiaminocarboxylic acid sulfate showed the presence of only one such group, which corresponds to structure (V) and not (VI).

<sup>1</sup> Mozingo, R., Wolf, D. E., Harris, S. A., and Folkers, K., unpublished data.



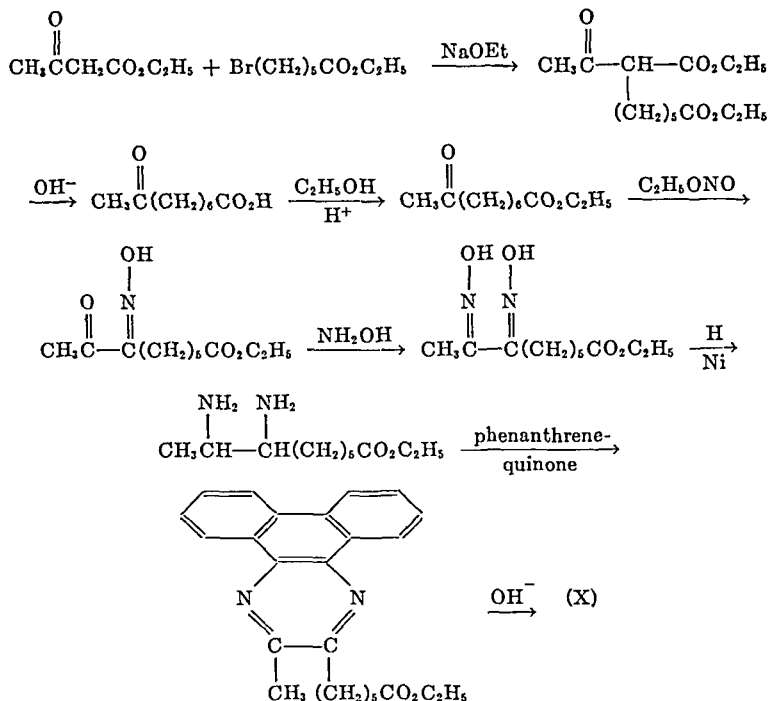
Oxidative cleavage reactions on the desthiiodiaminocarboxylic acid by nitric acid and alkaline permanganate gave low yields of mixtures of adipic and pimelic acids from which the pimelic acid was not separated satisfactorily on a micro scale. Oxidations with lead tetraacetate and alkaline hypochlorite solution were no better, but oxidation with alkaline periodate solution gave good yields of crude acid from which pure pimelic acid was obtained and identified as such and as its di-*p*-bromophenacyl ester by comparison with authentic samples of each.

When the desthiiodiaminocarboxylic acid was treated with phenanthrenequinone, the quinoxaline derivative (X) was obtained. Again (1), the oxidized form (X) rather than the dihydro form (XI) was obtained, as



shown by analyses and the formation of a characteristic red color with sulfuric acid. The compound showed no optical activity in NaOH solution, in agreement with structure (I) for biotin. The corresponding product from structure (II) would be expected to show optical activity. That the quinoxaline derivative had structure (X) was established by comparing it with an authentic specimen synthesized according to the accompanying reactions.

The quinoxaline derivatives of the synthetic and isolated diaminocarboxylic acids were synthesized to facilitate the comparison of the two compounds, since the quinoxaline derivatives contain no asymmetric carbon atoms. In this way the resolution of the synthetic diaminocarboxylic acid was obviated. The ultraviolet absorption spectrum of the synthetic compound was compared with the spectra of the quinoxaline and dihydroquin-



oxaline derivatives of 3,4-diaminotetrahydrothiophene (1). The curve of the synthetic compound was similar to that of the quinoxaline derivative of 3,4-diaminotetrahydrothiophene, in agreement with the other data that the synthetic compound was the oxidized form (X).

Therefore, the isolation of pimelic acid as the oxidation product of the desthiiodiaminocarboxylic acid and the synthesis of the quinoxaline derivative established structure (V) for the desthiiodiaminocarboxylic acid and established biotin as 2'-keto-3,4-imidazolido-2-tetrahydrothiophenevaleric acid, as represented by structure (I). In an accompanying paper (5) structure (I) has been established for biotin by direct demonstration of the presence of a 5-membered sulfur ring with an *n*-valeric acid side chain attached in the  $\alpha$  position.

#### EXPERIMENTAL

The biotin methyl ester (m.p. 162–163° corrected,  $[\alpha]_D^{25} = +55.5^\circ$ ) used in these studies corresponded exactly in properties to that isolated (6) by other methods.

*Desthiobiotin Methyl Ester* (Methyl (4-Methyl-5-imidazolidone-2)-caproate)—100 mg. of biotin methyl ester were dissolved in 100 cc. of 90 per cent ethanol and refluxed with approximately 5 gm. of Raney's nickel for 5 hours. The reaction mixture was centrifuged to remove the nickel, which was washed with 125 cc. of hot 95 per cent ethanol in five portions, then with 35 cc. of hot methanol. The combined eluates were concentrated to dryness *in vacuo* at not over 40°. The residue was taken into about 5 cc. of methanol, centrifuged, and the precipitate was washed with an additional 5 cc. of methanol. The methanol solution was concentrated *in vacuo* and the residue was thoroughly dried. Extraction of the dry material with chloroform gave a solution of the crude ester. Insoluble material was collected on a filter and the filtrate was concentrated *in vacuo*. The yield was 85.5 mg. Purification was accomplished by sublimation of the crude product at  $10^{-5}$  mm. and 100°. The highest melting point observed was 69–70°.  $[\alpha]_D^{28} = +2.6^\circ$  for a 2 per cent solution in chloroform.

$C_{11}H_{20}O_3N_2$ .	Calculated.	C 57.87, H 8.83, N 12.27
228.3	Found.	" 57.84, " 8.83, " 12.27

It was observed that desthiobiotin, desthiodiaminocarboxylic acid sulfate, and dihydrochloride had very low positive specific rotations. Even though the removal of the sulfur atom destroys the asymmetry at carbon atom 2 (I), there is no evidence that partial racemization took place at carbon atoms 3 and 4, although the possibility of such a reaction has been considered.

*Desthiodiaminocarboxylic Acid Dihydrochloride* ( $\xi, \eta$ -Diaminopelargonic Acid Dihydrochloride)—85 mg. of crude desthiobiotin ester were dissolved in 15 cc. of concentrated HCl and the solution was heated in a sealed tube at 200° for 1 hour. Some darkening occurred with the formation of an insoluble film on the surface of the solution. The insoluble material was collected on a filter and the filtrate was concentrated *in vacuo* to dryness and then twice concentrated with 1 cc. of water to remove traces of HCl. The crude product was dissolved in absolute ethanol, traces of insoluble material were collected on a filter, and the clear solution was concentrated *in vacuo* to dryness. Crystals were obtained by dissolving the residue in a minimum of methanol, and diluting with about 3 volumes of absolute ethanol and then with ether until cloudy. Small clumps of crystals of the diamine hydrochloride were obtained. The yield was 61 mg.; the melting point 180–182°.  $[\alpha]_D^{20} = +4.04^\circ$  for a 0.75 per cent solution in methanol.

$C_9H_{22}Cl_2N_2O_2$  (261.2). Calculated, C 41.38, H 8.49; found, C 41.36, H 8.66

*Desthiodiaminocarboxylic Acid Sulfate* ( $\xi, \eta$ -Diaminopelargonic Acid Sulfate)—20 mg. of desthiobiotin methyl ester were placed in each of eight

small test-tubes. To each were added 400 mg. of  $\text{Ba}(\text{OH})_2$  and 2 cc. of water. The tubes were sealed and placed in a bath at  $140^\circ$  for 16 to 17 hours. The contents of the tubes were combined, saturated with  $\text{CO}_2$ , and the barium carbonate was centrifuged and washed. The total aqueous portion was acidified with sulfuric acid until faintly acid to Congo red. The barium sulfate was separated and the filtrate was concentrated nearly to dryness *in vacuo*. Addition of methanol gave a crystalline product. The yield was 151.5 mg. The crude product was recrystallized by dissolving it in a minimum of water and diluting with about 3 volumes of methanol. The melting point was  $242\text{--}243^\circ$ .  $[\alpha]_D^{26} = +7.75^\circ$  for a 1.4 per cent solution in water.

$\text{C}_9\text{H}_{12}\text{N}_2\text{O}_4\text{S}$	Calculated.	C 37.75, H 7.75, N 9.79, C-methyl 5.25
286.4	Found.	" 37.71, " 7.82, " 9.78, " 1.88

*Oxidation of Desthiodiaminocarboxylic Acid Sulfate ( $\xi, \eta$ -Diaminopelargonic Acid Sulfate) with Periodate*—During the preliminary studies of oxidizing agents and conditions for this oxidation, use was made of the biological method for the determination of pimelic acid (7). In the first experiments, this bioassay indicated the presence of pimelic acid in the crude oxidation products, and in the later ones it aided in the selection of the oxidation conditions.

50 mg. of desthiodiaminocarboxylic acid sulfate were dissolved in 5.5 cc. of water. To the solution were added 1.15 cc. of  $N$   $\text{NaOH}$  and 2.55 cc. of 0.206  $N$  periodic acid and the mixture was allowed to stand overnight at room temperature. Tests with potassium iodide-starch paper after this period were positive. The mixture was warmed to  $40^\circ$  for 3 hours and finally to  $75^\circ$  for  $2\frac{1}{2}$  hours to complete the oxidation, or until tests for the oxidizing agent were no longer positive. The solution was acidified with  $\text{HCl}$  to Congo red and extracted continuously with ether. The extract yielded 22.7 mg. of white solid material. Preliminary purification was accomplished by sublimation in a high vacuum, the last sublimed portion melting at  $95\text{--}100^\circ$ .

The crude sublimes from the oxidation of three 50 mg. samples were recrystallized repeatedly from a mixture of ether and petroleum ether. In this way 17 mg. of nearly pure pimelic acid were isolated from the three sublimes. Recrystallization gave pure material melting at  $103\text{--}104^\circ$ . A mixture of this material with a known sample of pure pimelic acid (m.p.  $103\text{--}104^\circ$ ) melted at  $103\text{--}104^\circ$ .

$\text{C}_7\text{H}_{12}\text{O}_4$  (160.2). Calculated, C 52.49, H 7.56; found, C 52.71, H 7.72

For further identification the *p*-bromophenacyl ester was prepared. The pure ester melted at  $137.5\text{--}138.5^\circ$ . A mixture of the compound with a pure

known sample of *p*-bromophenacyl pimelate (m.p. 137–137.5°) showed no depression of the melting point.

$C_{23}H_{22}Br_2O_6$  (554.3). Calculated, C 49.84, H 4.00; found, C 50.14, H 4.24

By fractional crystallization from ether-petroleum ether a trace of crude adipic acid was obtained melting at 144.5–150°. The melting point of a mixture of this substance with adipic acid (m.p. 150.5–151°) was 148–150°.

*Dibenzoquinoxaline Derivative of Desthiodiaminocarboxylic Acid (2-Methyl-3-dibenzoquinoxalinecaproic Acid)*—50 mg. of desthiodiaminocarboxylic acid sulfate were converted to the free diaminocarboxylic acid by treatment with the calculated amount of  $Ba(OH)_2$ . The desthiodiaminocarboxylic acid was dissolved in 15 cc. of ethanol and 43 mg. of phenanthrenequinone were added to the solution. The solution was refluxed for 10 hours on the water bath. A small amount of insoluble material was removed by filtration and the filtrate was concentrated to 2 cc. *in vacuo*. On addition of 3 cc. of water a crystalline material separated. This was washed twice with an alcohol-water mixture and dried. The material, 47 mg., m.p. 182–186°, was recrystallized from alcohol-water. The recrystallized compound, 44 mg. of pale yellow micro plates, melted at 186–187°. With sulfuric acid the compound produced a deep red color. A 0.9 per cent solution of the compound in 0.04 *N* NaOH showed no optical activity in a 2 dm. tube.

$C_{23}H_{22}O_2N_2$ .	Calculated.	C 77.06, H 6.19, N 7.82
358.4	Found.	" 77.39, " 5.95, " 8.14

*$\eta$ -Ketopelargonic Acid*—The  $\epsilon$ -bromocaproic acid was prepared according to the general method of Marvel and coworkers (8). 133.9 gm. of ethyl acetoacetate were dissolved in ethanol to which 23.7 gm. of sodium had been added. A little sodium iodide was added and, after the solution had been heated to boiling, 250 gm. of ethyl  $\epsilon$ -bromocaproate were added. The solution was refluxed for several hours, the alcohol was distilled, and the product was dissolved in ether and was washed with water to free it from sodium bromide. The product was distilled under reduced pressure; b.p. 144–148° at 0.9 mm.; yield 188 gm. (64.5 per cent).

2.3 gm. of the ester were dissolved in diethylene glycol to which had been added 8 gm. of NaOH dissolved in the minimum quantity of water. This solution was warmed on the steam bath for 30 minutes and the precipitate of sodium carbonate was separated. The mixture was poured into acidified water and extracted with chloroform and benzene. The extract was dried, concentrated, and distilled; b.p. 135° at 0.9 mm.; m.p. 39–40°.

$C_9H_{16}O_3$  (172.2). Calculated, C 62.76, H 9.37; found, C 62.33, H 9.33

*Ethyl  $\zeta$ , $\eta$ -Dioximinopelargonate*—Since it was found that the above keto acid was partially esterified by the action of ethyl nitrite and HCl, it was

first esterified and then nitrosated. The acid was dissolved in an excess of ethanol and after the addition of 1 cc. of concentrated  $H_2SO_4$  the solution was refluxed for 4 hours. The solution was concentrated, washed with water, and distilled; b.p. 91–96° at 0.4 mm.

$C_{11}H_{20}O_3$  (200.3). Calculated, C 65.97, H 10.07; found, C 65.99, H 9.90

The keto ester was nitrosated by a procedure similar to a method described (9). 4 gm. of the keto ester were dissolved in a little ethanol and 1 drop of concentrated HCl was added. The solution was heated to 45–50° and 1.54 gm. of ethyl nitrite were added; the temperature was kept near 50°. The solution was allowed to stand until there was no longer a test for nitrite. It was then treated with 2 equivalents of hydroxylamine hydrochloride and 3 gm. of sodium acetate. After the solution had been heated for 30 minutes on the steam bath and diluted with water, the dioximine crystallized. It was recrystallized from benzene and then from methanol and water; m.p. 107–108°.

$C_{11}H_{20}O_4N_2$ .	Calculated.	C 54.08, H 8.25, N 11.47
244.3	Found.	" 53.68, " 7.89, " 11.52
		" 53.46, " 8.03

*Ethyl  $\zeta, \eta$ -Diaminopelargonate*—6.1 gm. of the dioximino ester were dissolved in 50 cc. of methanol and 100 cc. of liquid ammonia and hydrogenated over 3 gm. of Raney's nickel at 50–55° and 140 atmospheres for about 2 hours. After removal of the catalyst by filtration, the ammonia was removed by concentration under reduced pressure. The residue (3 gm.) was dissolved in 50 cc. of ethanol and treated with sulfuric acid until just acid to Congo red, when the sulfate crystallized; m.p. 274° with decomposition.

$C_9H_{23}O_5N_3S$ .	Calculated.	C 42.02, H 8.34, N 8.91
285.4	Found.	" 41.48, " 8.12, " 9.13

*Ethyl 2-Methyl-3-dibenzoquinoxalinecaproate*—0.005 mole of ethyl  $\zeta, \eta$ -diaminopelargonate was refluxed overnight with 0.52 gm. of phenanthrenequinone in 16 cc. of ethanol. The solution was filtered and concentrated to dryness. The gummy residue was crystallized from alcohol and water; m.p. 78–79°. The crystals gave a red color with sulfuric acid.

$C_{26}H_{28}N_2O_2$ .	Calculated.	C 77.69, H 6.77, N 7.25
386.5	Found.	" 77.42, " 6.89, " 7.55

*2-Methyl-3-dibenzoquinoxalinecaproic Acid*—This acid was prepared by the hydrolysis of the ethyl ester (60 mg.) with 1 equivalent of NaOH in water, and by the direct condensation of the  $\zeta, \eta$ -diaminopelargonic acid with phenanthrenequinone. After crystallization from ethanol the melt-

ing point of the compound was 186–187°. A mixture of this compound with the quinoxaline derivative of the desthiobiotin prepared from biotin melted at 186–187°.

$C_{23}H_{22}N_2O_2$ .	Calculated.	C 77.06, H 6.19, N 7.82
358.4	Found.	" 77.05, " 6.28, " 8.18

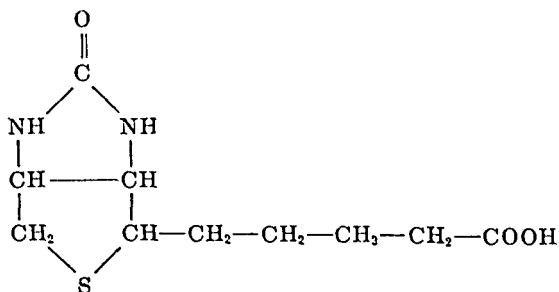
The coworkers at Cornell University Medical College wish to express their appreciation to Dr. J. R. Rachele for microanalyses and to Miss Eleanor Hague for the pimelic acid assays.

The coworkers in the Merck Research Laboratory wish to acknowledge the valuable assistance of Mr. Rickes and Mr. Chalet on the isolation of biotin, Dr. J. L. Stokes and assistants for microbiological assays, Mr. Anderson and Mr. Easton on synthetic work, Mr. Hayman, Mr. Clark, and Mr. Boos for microchemical analyses, and Mr. Bastedo, Jr., for the ultraviolet absorption determination on the 2-methyl-3-dibenzoquinoxalinecaproic acid.

#### SUMMARY

Treatment of biotin with Raney's nickel catalyst cleaves the sulfur atom and 2 atoms of hydrogen are added. Hydrolysis of this desthiobiotin in acid or alkaline solution gave the corresponding desthiodiaminocarboxylic acid which on oxidation with periodate yielded a dibasic acid identified as pimelic acid. By treatment with phenanthrenequinone the desthiodiaminocarboxylic acid was converted to the corresponding quinoxaline derivative which agreed in all its properties with the synthetically prepared compound.

The formation of pimelic acid by oxidation of desthiobiotin and the identity of the synthetic quinoxaline derivative with that obtained from desthiobiotin, in conjunction with other published data, establishes the structure of biotin as given in the accompanying formula.



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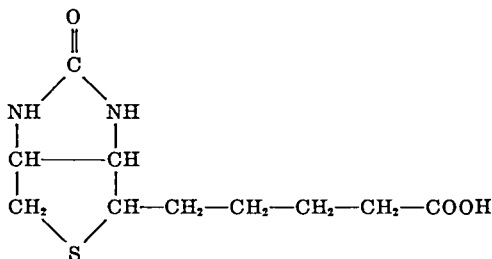
# THE STRUCTURE OF BIOTIN: THE FORMATION OF THIOPHENEVALERIC ACID FROM BIOTIN\*

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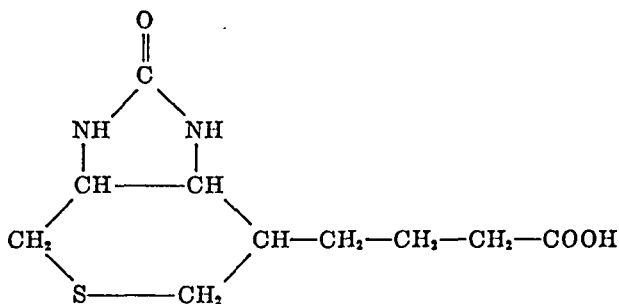
Studies on the structure of biotin,  $C_{10}H_{16}O_3N_2S$ , have led us to the conclusions that biotin contains a cyclic urea structure (1, 2), that the sulfur is present in a thio ether linkage (1), and that the carboxyl group of biotin is present most probably in an *n*-valeric acid side chain (3). Furthermore we have recently presented evidence to show that the urea ring is 5-membered, and that each of the carbon atoms attached to the amino groups in the diaminocarboxylic acid derived from biotin also carries a hydrogen atom (4). On the basis of these data we have suggested that the most likely



(I)

structure of biotin is that expressed by formula (I). Evidence for the presence of an *n*-valeric acid side chain in biotin was based on the isolation of adipic acid as one of the oxidation products of the diaminocarboxylic acid derived from biotin (3). However, as we have pointed out (3), if the adipic acid were to arise through the decarboxylation of an intermediary malonic or  $\beta$ -keto acid formed during the oxidation, then another structure would be possible, as expressed by formula (II).

\* The authors wish to express their appreciation to the S. M. A. Corporation for a research grant which has aided greatly in this work. They also wish to thank Mr. W. O. Frohring and the Research Staff of the S. M. A. Corporation and Dr. R. Major and the Research Staff of Merck and Company, Inc., for supplies of biotin.



(II)

It is the purpose of this paper to describe experiments which offer direct proof of the existence of a 5-membered sulfur ring in biotin with an *n*-valeric acid side chain attached in the  $\alpha$  position, thus affording conclusive evidence that formula (I) represents the correct structure of biotin. In an accompanying paper the same conclusion is arrived at through a study of desthiobiotin (5).

As we have earlier stated, we suspected that the diaminocarboxylic acid derived from biotin was a 3,4-diaminotetrahydrothiophene derivative. It was realized that if this were true one might be able through degradation to arrive at a thiophene derivative which could readily be met by direct synthesis to establish its structure. We early investigated exhaustive methylation as a possible method of approach. Preliminary experiments with methyl iodide and with dimethyl sulfate yielded no isolable products. When larger amounts of biotin became available, methylation of the diaminocarboxylic acid with dimethyl sulfate and alkali was tried on a larger scale. We were unable to isolate any crystalline methylation product, and decomposition of the methylation mixture under various conditions of temperature and alkalinity caused the liberation of much trimethylamine but produced little or no material of the desired physical and chemical properties. However, it was found that methylation mixtures which had been acidified with HCl and refluxed contained small amounts of an ether-soluble oil which on distillation yielded an acidic, sulfur-containing crystalline fraction. In this way, from 50 mg. samples of the diaminocarboxylic acid sulfate we have obtained each time approximately 1 mg. of crystals, m.p. 40–41°. Variations in the conditions of methylation did not increase the yield. It is possible that methylation of the thio ether to a methyl sulfonium derivative may have occurred, and decomposition of this derivative might take place in several ways. In this respect it is of interest that the methyl sulfonium base of  $\alpha$ -methyl tetrahydrothiophene readily undergoes ring fission (6).

On the hypothesis that formula (I) expresses the correct structure for biotin, and that therefore the expected product from an exhaustive methyl-

ation would be  $\delta$ -( $\alpha$ -thienyl)-valeric acid, we synthesized this acid from thiophene and glutaric anhydride by the method used by Fieser and Kennelly (7) to synthesize  $\gamma$ -( $\alpha$ -thienyl)-butyric acid. The position of the side chain was shown, as indicated in the accompanying equations, by oxidation

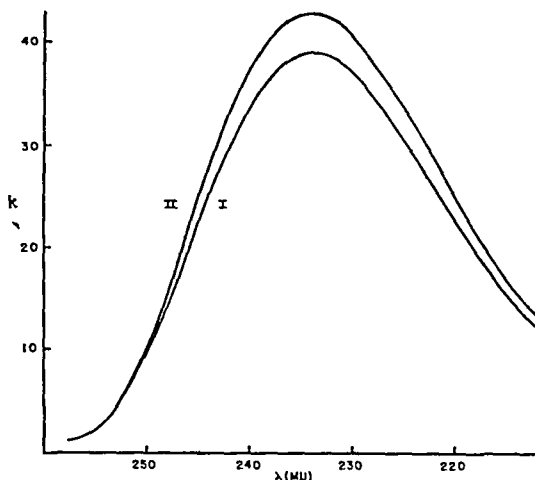
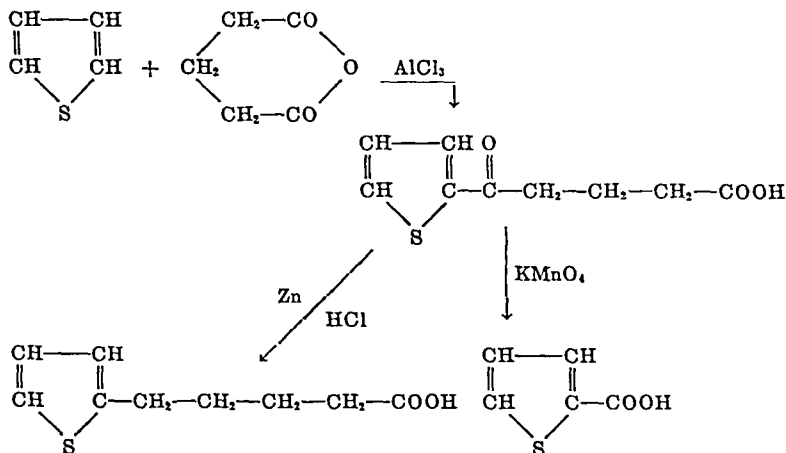


FIG. 1. Ultraviolet absorption spectra of (Curve I) the compound isolated from biotin, (Curve II) synthetic  $\delta$ -( $\alpha$ -thienyl)-valeric acid in 95 per cent alcohol. The wave-length is plotted against the specific extinction,  $k$  ( $k = E/cd$ , where  $E$  = extinction,  $c$  = concentration in gm. per liter, and  $d$  = cell thickness in cm.).

of the intermediary  $\gamma$ -( $\alpha$ -thenoyl)-butyric acid to  $\alpha$ -thiophenic acid which melted at 125–126°.



The synthetic  $\delta$ -( $\alpha$ -thienyl)-valeric acid showed the same crystalline form, solubilities, and melting point ( $40-41^\circ$ ) as the compound isolated from the methylation of the diaminocarboxylic acid from biotin. A mixture of the isolated and synthetic compounds showed no depression of the melting point. Both compounds gave a deep blue-green color with Liebermann's nitrite-sulfuric acid reagent, and a slowly forming purple color with isatin-sulfuric acid reagent. An ultraviolet absorption study in the range 212 to 400  $m\mu$  showed a single absorption peak at 234  $m\mu$  in both cases, as shown in Fig. 1. Carbon-hydrogen analyses on the isolated material were in agreement with the calculated values for  $\delta$ -( $\alpha$ -thienyl)-valeric acid. All these data demonstrate that the isolated compound is  $\delta$ -( $\alpha$ -thienyl)-valeric acid. The conditions under which this compound is formed from the diaminocarboxylic acid derived from biotin minimize the possibility of its formation through any deep seated rearrangement. The formation, therefore, of  $\delta$ -( $\alpha$ -thienyl)-valeric acid from biotin, in conjunction with our other published data, justifies the conclusion that the structure expressed by formula (I) represents the structure of biotin.

#### EXPERIMENTAL

*Methylation of Diaminocarboxylic Acid*—50 mg. of diaminocarboxylic acid sulfate (1) were dissolved in 1 cc. of water and the solution was made alkaline to phenolphthalein by the addition of 0.5 cc. of 6.8 per cent KOH solution. There were then added with stirring 245 mg. of dimethyl sulfate in sixteen portions, along with 1.6 cc. of KOH solution in sixteen portions. The clear solution was acidified by the addition of 2 cc. of concentrated HCl and was refluxed for 2 hours. The solution on cooling became slightly turbid and the reflux condenser contained small amounts of ether-soluble material. The apparatus was washed out with ether and the aqueous solution was extracted continuously with ether for 2 hours. The ether extract contained 2 mg. of a yellow oil which was distilled under an oil pump vacuum. At  $55^\circ$  a colorless liquid distilled which on cooling crystallized in long needles. The yield was approximately 1 mg., m.p.  $40-41^\circ$ . A deep blue-green color was produced by treatment of the compound with Liebermann's nitrite-sulfuric acid reagent. With isatin-sulfuric acid reagent a slowly forming purple color was produced. A qualitative test showed sulfur to be present.

$C_9H_{12}O_2S$  (184.2). Calculated, C 58.70, H 6.52; found, C 58.99, H 6.83

*Synthesis of  $\delta$ -( $\alpha$ -Thienyl)-valeric Acid*—The method used by Fieser and Kennelly (7) for the preparation of the lower homologue of this acid was used. From the Friedel-Crafts reaction, with 20 gm. of thiophene and 25 gm. of glutaric anhydride, we obtained 12 gm. of  $\gamma$ -( $\alpha$ -thenoyl)-butyric

acid, m.p. 92–94°. Reduction of 9 gm. of this keto acid with Zn and HCl yielded a yellow oil. Distillation of the oil at 145–150° and 3 mm. gave 3 gm. of a colorless liquid which crystallized in long needles on cooling. Redistillation of this compound yielded pure  $\delta$ -( $\alpha$ -thienyl)-valeric acid, m.p. 40–41°. The neutral equivalent found was 182, in agreement with the calculated value of 184.

$C_9H_{12}O_2S$ .	Calculated.	C 58.70, H 6.52, S 17.41
184.2	Found.	" 58.90, " 6.58, " 17.22

100 mg. of the  $\gamma$ -( $\alpha$ -thenoyl)-butyric acid were oxidized with alkaline permanganate by the method of Voerman (8). The oxidation product was crystallized three times from water and sublimed *in vacuo*. The melting point of the sublimate (125–126°) agreed with that given in the literature for  $\alpha$ -thiophenic acid (126.5°).

The color reactions of the synthetic  $\delta$ -( $\alpha$ -thienyl)-valeric acid with Liebermann's reagent and with isatin-sulfuric acid were the same as those observed with the compound isolated from biotin. A mixture of the synthetic  $\delta$ -( $\alpha$ -thienyl)-valeric acid with the compound isolated from biotin melted at 40–41°.

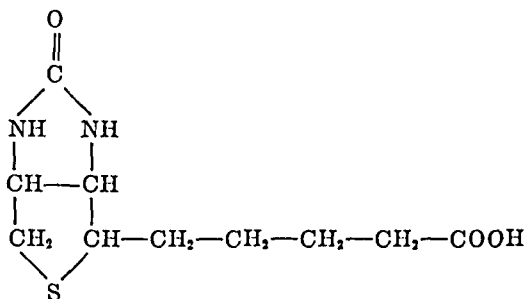
*Ultraviolet Absorption Spectra*—The ultraviolet absorption characteristics of the synthetic  $\delta$ -( $\alpha$ -thienyl)-valeric acid and of the compound isolated from the methylation experiment were studied in the region 212 m $\mu$  to 400 m $\mu$  with the aid of a Beckman quartz spectrophotometer. A distilled sample of the isolated compound weighing 0.173 mg. was used for this purpose. The solvent used was 95 per cent ethanol and the temperature was about 21°. The concentrations of the isolated and synthetic samples were 0.00692 and 0.00586 gm. per liter, respectively. The cell thickness was 1 cm. Both compounds showed an absorption peak at 234 m $\mu$ . In Fig. 1 the specific extinction coefficients (*i.e.*, extinction per unit concentration and unit cell thickness) of both compounds are plotted against the wave-length in the range of the absorption peak. The difference in the heights of the absorption peaks may be due in large part to the experimental error in weighing the small sample of isolated compound, and also the possible presence of small amounts of impurities.

The authors wish to express their appreciation to Dr. Julian R. Rachele of this laboratory for carrying out the microanalyses.

#### SUMMARY

Methylation of the diaminocarboxylic acid from biotin with dimethyl sulfate and alkali, and treatment of the product with hydrochloric acid, results in the formation of  $\delta$ -( $\alpha$ -thienyl)-valeric acid, identical in all its properties with the synthetically prepared compound.

The formation of this compound from a degradation product of biotin, in conjunction with our other published data, establishes the accompanying structure for biotin, 2'-keto-3,4-imidazolido-2-tetrahydrothiophenevaleric acid.



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# THE BENZOYLATION AND RESOLUTION OF ALANINE

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Fischer (3) resolved synthetic alanine in the form of its benzoyl derivative. Recently several publications have contained comments on the benzylation and resolution of this amino acid (1, 2, 5). Our interest, arising from the need for synthetic substrates for use with chick embryo extracts (4), resulted in the modifications of the preparation of benzoyl-alanine and in certain observations on the optical activities of the products recorded below.

*Benzoylation of dl-Alanine*—Benzoylation of alanine has been accomplished in the presence of excess  $\text{NaHCO}_3$  (3, 5) as well as in the presence of excess  $\text{NaOH}$  (1) with benzoyl chloride as the acylating reagent. Addition of  $\text{HCl}$  precipitated a mixture of benzoic acid and benzoylalanine from which the benzoic acid was removed by extraction with petroleum ether.

We have found it convenient to use theoretical quantities of  $\text{NaOH}$  and to increase the concentration of reactants during benzylation. By using known amounts of alkali, most of the benzoic acid may be precipitated by addition of the calculated quantity of  $\text{HCl}$ , leaving the benzoylalanine in solution as the  $\text{Na}$  salt. A typical protocol of preparation follows.

Synthetic alanine (22.5 gm., 0.25 mole) was dissolved in  $\text{NaOH}$  (62.5 ml. of 4.00  $\text{N}$ , 0.25 mole) and placed in a 500 ml. three-necked flask fitted with a stirrer and thermometer. The flask was immersed in an ice-salt bath until the temperature dropped to  $3^\circ$ . Benzoyl chloride (0.5 mole) and  $\text{NaOH}$  (187.5 ml. of 4.00  $\text{M}$ , 0.75 mole) were added alternately in ten equal instalments each, so that the temperature remained below  $20^\circ$ . This required approximately 5 minute intervals. Stirring was continued an hour longer. The solution now contained, by calculation, 0.25 mole of  $\text{Na}$  benzoylalanine, 0.25 mole of  $\text{Na}$  benzoate, and 0.5 mole of  $\text{NaCl}$ . Hydrochloric acid (62.5 ml. of 4.0  $\text{M}$ , 0.25 mole) was added and the mixture kept at  $5^\circ$  overnight. The precipitate of benzoic acid weighed 22 gm. and had a titration equivalent of 125 (theory, 30 gm., equivalent 122). The mixture of filtrate and washings (total volume 450 ml.) was heated to boiling and an excess of concentrated  $\text{HCl}$  was added to the hot solution. After being cooled overnight at  $5^\circ$  the precipitate was collected, washed with cold water, and dissolved in 300 ml. of hot water containing 0.225 mole of  $\text{NaOH}$ . After cooling to  $5^\circ$ , a slight precipitate was removed and the filtrate was heated



and then acidified to Congo red. After 18 hours refrigeration, the precipitate was collected and dried at 100°. It weighed 43 gm. and had a titration equivalent of 183 (theory, 48 gm., equivalent 192). One recrystallization from 500 ml. of hot water gave 38 gm. (79 per cent) of material with the theoretical titration equivalent and accepted melting point.

*Resolution of dl-Benzoylalanine*—Our experience with the resolution of benzoylalanine by means of brucine and strychnine agrees with that of Dunn (2). The scheme proposed and used by Pope and Gibson (6) is as effective and more economical of alkaloids than that used by Pacsu and Mullen (5).

*Optical Activity of Benzoylalanines*—Because of the low solubility of benzoylalanine in water (about 1 gm. per 100 ml.) the specific rotation is taken after the acid has been dissolved in an equivalent of alkali. Pacsu and Mullen (5) state that the value of  $[\alpha]_D$  varies with the concentration

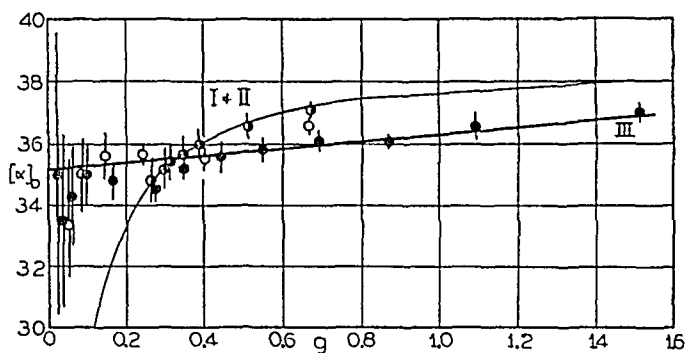


FIG. 1. Specific rotation and concentration of benzoylalanine in alkali. Solvent, ● equivalent NaOH, ○ equivalent KOH, ● equivalent KOH, data of Pacsu and Mullen (5). The vertical line through each point indicates the uncertainty of  $[\alpha]_D$ . Curves I, II, and III are plotted from Equations I, II, and III.

of benzoylalanine. In their Table I, figures are given which purport to show that  $1/\log [\alpha]_D$  is a linear function of  $1/g$  where  $g$  represents the gm. of benzoylalanine present as potassium salt in 10 ml. of solution. Evidently another relationship is considered valid too, for in the foot-note to their Table I they state, "The rotation of a quantity ( $g$ ) of benzoylalanine is calculated from the equation,  $n = (3.700 - 1/g)/0.095$ , where  $n$  represents the change of rotation in 0.1° units over the value  $[\alpha]_D = 34.8^\circ$ ." The two relationships may be transformed by appropriate algebraic manipulations into the equations

$$[\alpha]_D = 38.7 - 1.05/g \quad (I)$$

$$1/\log [\alpha]_D = 0.6290 + 0.0053/g \quad (II)$$

In spite of the evident mathematical incongruity, both equations fit the data of Pacsu and Mullen (5) very well. In fact, the fit is considerably

better than would be expected from the uncertainty of  $0.02^\circ$  usually ascribed to polarimeter readings. Our own measurements over a wider range of concentrations fit neither of these equations, and we feel that both should be discarded.

In Fig. 1, three sets of data are plotted. These are (1) the data of Pacsu and Mullen as set forth in their Table I, (2) a similar set obtained with our own material, in which each solution was made independently with KOH as the alkali, and (3) a more extensive set of data obtained from solutions prepared by successive dilutions of the most concentrated one and in which the alkali was NaOH. A vertical line is drawn through each point to indicate the uncertainty of  $[\alpha]_D$  resulting from the uncertainty of  $\pm 0.02^\circ$  in  $\alpha$ . The tubes used were 0.5, 1.0, and 2.0 dm. in length, depending on the concentrations of the solutions. A calculated line for Equations I and II and a straight line which we think best represents the data are drawn. The equation of the straight line is

$$[\alpha]_D = 35.2 + 1.0 g \quad (\text{III})$$

The temperature in our experiments was  $22-25^\circ$ ; presumably Mullen and Pacsu worked at nearly the same range, although the temperature is not stated.

*Optically Active Alanines*—The hydrolysis of the optically active benzoylalanines and isolation of the *d*- and *l*-alanines were conducted essentially as indicated by Pacsu and Mullen (5). Dunn (2) quotes several sources to indicate that the optical activity of alanine in  $N$  HCl is considerably higher than the figure reported by Pacsu and Mullen ( $\pm 10.3^\circ$ ). From the data which they give we calculate  $\pm 14.55^\circ$  in agreement with our own value and that of others quoted by Dunn (2). The figure given by Pacsu and Mullen results if in the calculation the weight of alanine hydrochloride formed is used instead of the weight of alanine dissolved.

#### SUMMARY

A modified method of preparing and purifying *dl*-benzoylalanine is given. The optical activity of benzoylalanine in an equivalent of alkali varies with the concentration  $g$  (in gm. per 10 ml.) according to the equation  $[\alpha]_D = 35.2 + 1.0 g$ . The optical activity of alanine in excess HCl is  $[\alpha]_D^{20} = 14.5^\circ$ , the sign of the rotation being opposite in sense to the configuration.

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# THE FATE OF OXALIC ACID ADMINISTERED TO THE RAT\*

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Recent reports showing the presence of oxalic acid in spinach and other leaf vegetables (10, 11, 16, 17) have created an interest in the behavior of oxalic acid in metabolism. Some study has been made of the influence of calcium oxalate and of spinach in the diet upon calcium metabolism (2, 3, 6, 7, 11, 13). Experiments recording the excretion of oxalic acid after administration of sodium oxalate or oxalic acid have been reported for the dog (14), for the guinea pig (4), for the rabbit (8, 9), and for man (9), with rather wide variations, however, in the results reported. We are recording here observations on the manner in which oxalic acid is excreted in the rat when oxalate is administered *per os* and also when injected subcutaneously.

## EXPERIMENTAL

Six albino rats from the Yenching stock colony weighing about 200 gm. were placed in the glass jar metabolism cages used in previous experiments (1), cages designed to facilitate a clear cut separation of urine and feces. All metabolism periods were 4 days in length. The experiment was extended over fifteen consecutive 4 day periods. The basal diet, shown in Table I, was low in calcium and phosphorus and was fed as a control diet in Period 1. It was made of a pasty consistency to avoid scattering. This same diet had also been fed for a 4 day preexperimental period. In the succeeding periods, this diet was supplemented (Period 2) by calcium lactate plus sodium oxalate in equivalent proportions (Diet 1), and (Period 6) by sodium oxalate alone (Diet 2), and finally (Period 10) by injecting subcutaneously 1 ml. of solution daily, containing 27.2 mg. of sodium oxalate, the injection, however, being limited to the first 2 days of this period. Our previous experience had shown that rats on a calcium-free diet could be fed as much as 100 mg. of sodium oxalate without toxic effects. The rats, with the exception of one animal which died in Period 10, remained healthy throughout the experiment and continued to gain in weight.

At the end of each 4 day period the glass cage was washed twice with distilled water acidified with hydrochloric acid and these washings were added to the urine, the total volume being kept within 100 ml. The feces for each period were dried at 55–60° for 24 hours and the dried weight re-

\* Aided by a grant from the Ella Sachs Plotz Foundation.

corded. They were then ground in a micro mill to pass a 40 mesh sieve and preserved for analysis. Aliquots of both urine and feces were analyzed for calcium and for oxalic acid. For calcium, the samples were ashed in silica dishes and the determination made by the method of Kramer and Tisdall (12). The oxalic acid was determined as indicated below.

*Method for Oxalic Acid*—After an extended study of the various methods proposed and their modifications, the method of Dakin (5), involving extraction of the oxalic acid with ether, was adopted. An extraction apparatus similar to that recommended by Palkin *et al.* (15) was employed, designed to extract 5 ml. of the liquid sample. Urine samples were deproteinized with trichloroacetic acid, and calcium added to precipitate the oxalate, which was dissolved in hydrochloric acid and added to the extractor. Separations in each case were made by the centrifuge instead

TABLE I  
*Composition of Diets*

	Basal diet	Diet 1	Diet 2
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Starch.....	47.5	47.5	47.5
Cellulose.....	2.5	2.5	2.5
Casein*.....	12	12	12
Lard.....	25	25	25
Salt Mixture 4†.....	2	2	2
Cod liver oil.....	5	5	5
Yeast.....	5	5	5
Sodium oxalate.....	0	1.31	1.31
Calcium lactate.....	0	3.00	0
Oxalic acid content.....	0	0.88	0.88
Calcium content.....	0.085	0.475	0.085

\* Glaxo Laboratories, casein A.

† NaCl 105, KCl 290, MgSO<sub>4</sub> 90, FeSO<sub>4</sub>·7H<sub>2</sub>O 18, NaF 0.57, CuSO<sub>4</sub>·5H<sub>2</sub>O 0.39, MnSO<sub>4</sub> 0.20, KAl(SO<sub>4</sub>)<sub>2</sub>·12H<sub>2</sub>O 0.09, KI 0.05.

of filtering. Feces samples were treated with 2 N hydrochloric acid for about 18 hours with frequent stirring, and the clear solution was placed in the extraction apparatus. The ether in the extractor was made slightly alkaline with sodium hydroxide. About 5 hours were usually required for complete extraction. 3 ml. of water were then added and the ether was distilled off; the oxalate was finally precipitated as calcium oxalate and determined in the same manner as the calcium above. Control determinations with 2 to 3 mg. quantities of oxalic acid both in water solution and when added to urine and feces showed consistent recoveries of  $100 \pm 3$  per cent. To prevent decomposition of the oxalic acid in the metabolism cages, a small amount of toluene was added to the urine. Control experiments with the empty cages to which were added known amounts of oxalate showed no evidence of decomposition of oxalic acid in the cages.

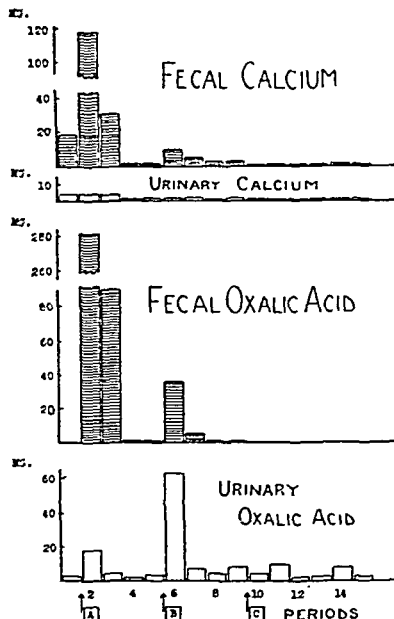


FIG. 1. Partition of exogenous oxalic acid for one rat, in 4 day periods. The rat received the following as indicated at A, B, and C respectively: Period 2, Diet 1, containing Ca + sodium oxalate (= 495 mg. of oxalic acid); Period 6, Diet 2, containing sodium oxalate only (= 253 mg. of oxalic acid); Period 10, the basal diet + sodium oxalate by subcutaneous injection (= 36 mg. of oxalic acid). In the other periods the basal diet only was fed.

TABLE II

*Average Recovery of Oxalic Acid after Administration of Sodium Oxalate*

Each period is 4 days in length; the oxalate intake is recorded in terms of its equivalent in oxalic acid.

Period No.	Diet*	No. of rats	Oxalic acid intake mg.	Oxalic acid recovered					
				Urine		Feces		Total	
				mg.	per cent	mg.	per cent	mg.	per cent
1	Basal diet	6	0	3.9		0		3.9	
2-5	Diet 1 (containing Ca and oxalate)	6	406	16†	3.9	325	80.0	341	84.0
6-9	Diet 2 (containing oxalate)	6	208	60†	29.0	40	19.0	100	48.0
10-15	Basal diet (+ sodium oxalate, injected)	5	36.5	15.4†	42.4	0		15.4	42.4

\* Diet 1 was fed in Period 2 only, followed by the basal diet in Periods 3 to 5. Diet 2 was fed in Period 6 only, followed by the basal diet in Periods 7 to 9. Sodium oxalate was injected on first 2 days only of Period 10.

† Exogenous oxalic acid only; this figure represents total oxalic acid less that excreted on the basal diet.

## Results

The complete metabolic data for one of the animals are shown in Fig. 1, which is typical of the results obtained for all six animals. All data on the oxalate metabolism are calculated in terms of oxalic acid. In Tables II and III are assembled the summarized metabolism data for both oxalic acid and calcium.

TABLE III

*Average Recovery of Calcium after Administration of Sodium Oxalate, for Experimental Periods and Diets As Indicated in Table II*

Period No.	Calcium intake*	Calcium recovered						Calcium retained	
		Urine†		Feces		Total			
	mg.	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
1	4.6	2.9		3.7		6.6		-2.0	
2-5	219.0	9.8	4.5	128.2	58.5	138.0†	63.0	+80.1	37.0
6-9	20.1	6.3		14.6		20.9§		-0.8	

\* Represents calcium in the basal diet except in Period 2, when Diet 1 was fed, containing 3 per cent of calcium lactate.

† Includes "endogenous calcium excretion."

‡ This figure may be compared with the figure 325 mg., the amount of oxalic acid found in feces (Table II)  $\approx$  144 mg. of Ca.

§ This figure may be compared with the figure 40 mg., the amount of oxalic acid found in feces (Table II)  $\approx$  17.5 mg. of Ca.

## DISCUSSION

The data show an average excretion of 3.9 mg. of oxalic acid per 4 day period (approximately 1 mg. per day) for the 200 gm. rat when fed an oxalate-free diet. We were, however, primarily interested in the extent to which oxalic acid can be recovered in the animal organism and the question of the fate of oxalic acid when fed or injected. The amount of oxalate, reckoned as oxalic acid, which we have administered for this purpose was from 20 to 100 times the amount of this endogenous excretion.

It is evident from the results obtained that the partition of exogenous oxalic acid between urine and feces depends primarily on the calcium content of the diet, calcium tending to detoxicate oxalic acid fed *per os* by forming insoluble calcium oxalate which is then excreted via the colon. Our experiments indicate that when equivalent amounts of calcium and oxalic acid are fed, 80 per cent of the oxalic acid can be accounted for in the feces, largely as calcium oxalate. Kohman (11) feeding calcium oxalate recovered from 34 to 73 per cent in the feces.

When rats were fed sodium oxalate with a low calcium diet, we were able to account for a total of 48 per cent of the oxalate fed, 29 per cent being

found in the urine. Among recent experiments on the feeding of oxalic acid with various types of diets are those of Orzechowski and coworkers (14) who report a recovery in the urine of 80 per cent in the dog, Borgström (4) with recoveries of 14 and 35 per cent in guinea pigs, Fraschenträger and Müller (8) who recovered 30 per cent in a rabbit, and Herkel and Koch (9) who fed 0.5 gm. to four human subjects and recovered about 5 per cent in the urine. We are omitting mention of data from experiments reported in the earlier literature, in many of which less accurate methods for the determination of oxalic acid were used or in which complicated diets of unknown calcium content were employed.

In no case have we obtained a complete recovery of the oxalic acid fed. It would appear then that some oxalic acid disappears in the body and this could be explained as due either to the destructive action of microorganisms in the intestine or to a definite oxidation within the body. It was to eliminate this first factor that we injected the oxalate, with the results indicated in Table II, Periods 10 to 15. To provide for the possibility that a portion of the oxalic acid might be temporarily retained in the body and excreted very slowly, we continued the collection of urine and determination of its oxalic acid content for a total of six periods (24 days), but were able to account in the urine for an average of only 42 per cent of that injected. Borgström (4) and Fraschenträger and Müller (8) likewise injected oxalic acid, using guinea pigs and rabbits, and they report recoveries of 96 and 77 per cent in the urine, respectively. Herkel and Koch (9), on the other hand, in experiments with rabbits find that the injected oxalic acid reappears only in part in the urine if at all. Our results suggest that in the rat the organism is able to utilize or otherwise dispose of part of the oxalic acid administered.

It may be noted from the data obtained (Table II, Periods 2 to 5) that, even when calcium is fed in a quantity equivalent to the amount of oxalate in the diet, an appreciable portion of the oxalic acid may be absorbed. It is shown furthermore that on an oxalate-free diet (Period 1) there is a small and fairly regular endogenous excretion of oxalic acid in the urine and none in the feces. When oxalic acid is either fed or injected, excretion of urinary calcium, as shown in Fig. 1, is reduced to a low and rather definite level. Apparently there is no danger, as suggested by Kohman (11), of removing calcium from the organism when oxalic acid is included in the intake.

#### SUMMARY

Sodium oxalate has been administered both orally and by injection to rats fed a synthetic diet and the partition of the oxalic acid between urine and feces determined. The endogenous excretion of oxalic acid in the rat is about 1 mg. per day.



Oxalic acid fed *per os* may be absorbed even in the presence of calcium, the amount absorbed being greater on a low calcium diet. When sodium oxalate was fed, an average of 29 per cent of the oxalic acid was recovered in the urine.

When injected, an average of 42 per cent was excreted in the urine. In no case was the recovery of the oxalic acid complete. It is suggested that in the rat the organism is able to oxidize or otherwise dispose of part of the oxalic acid administered.

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# THE EFFECT OF DICARBOXYLIC ACID ADMINISTRATION UPON THE EXCRETION OF TYROSINE METABOLITES BY THE GUINEA PIG

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Demonstration of the dependence of phenylalanine and tyrosine metabolism upon an adequate supply of ascorbic acid (1, 2) necessitated determining whether a similar relationship existed with other amino acids. Consequently tryptophane and glutamic acid were fed to guinea pigs receiving a vitamin C-free basal diet. When continued feeding failed to produce excretion of the corresponding keto acids or other metabolites of like nature, the glutamic acid supplement was replaced with tyrosine for control purposes. In contrast to earlier experiments with the latter amino acid there was observed a delay in metabolite excretion several days longer than any previously encountered. The possibility that the delay was in some way related to the previous intake of glutamic acid was readily investigated by the administration of a single dose of glutamic acid to guinea pigs exhibiting the characteristic urinary picture resulting from daily tyrosine supplementation. Without exception the administration of the dicarboxylicamino acid caused a prompt decrease in the excretion of *p*-hydroxyphenylpyruvic and homogentisic acids as does ascorbic acid.

Of several explanatory hypotheses which suggested themselves, the majority were easily eliminated. For example, the possibility of glutamic acid through the transamination reactions causing subsequent excretion of tyrosine rather than its  $\alpha$ -keto acid derivative was eliminated by the simple expedient of determining the total tyrosine value of the urine sample. Another hypothesis was derived from the known rôle of glutamic acid, as ketoglutaric acid, in the Szent-Györgyi-Krebs oxidation-reduction cycle. That such a system may take part in the catabolic metabolism of the aromatic amino acids is entirely within reason. Indeed in this instance it appeared that the dicarboxylic acid system might even be operating in conjunction with ascorbic acid, for as the experiments continued, and the animals became more deficient, repeated doses of glutamic acid were less effective.

The subsequent testing of the hypothesis described in this paper was accomplished by utilizing other members of the Szent-Györgyi-Krebs cycle as well as compounds of similar structure.

## EXPERIMENTAL

The vitamin C-free basal diet of Purina chow (complete ration) and the methods of feeding the guinea pigs and analyzing the urine samples are the same as those previously described (3). For the sake of brevity the majority of the results are reported in terms of daily excretion of *p*-hydroxyphenylpyruvic acid, since the homogentisic acid and total tyrosine excretion values paralleled those of the former compound.

In Fig. 1 representative results obtained with single doses of glutamic acid are presented. At first glance the effectiveness of glutamic acid in decreasing the excretion of the tyrosine metabolites appears to be quite

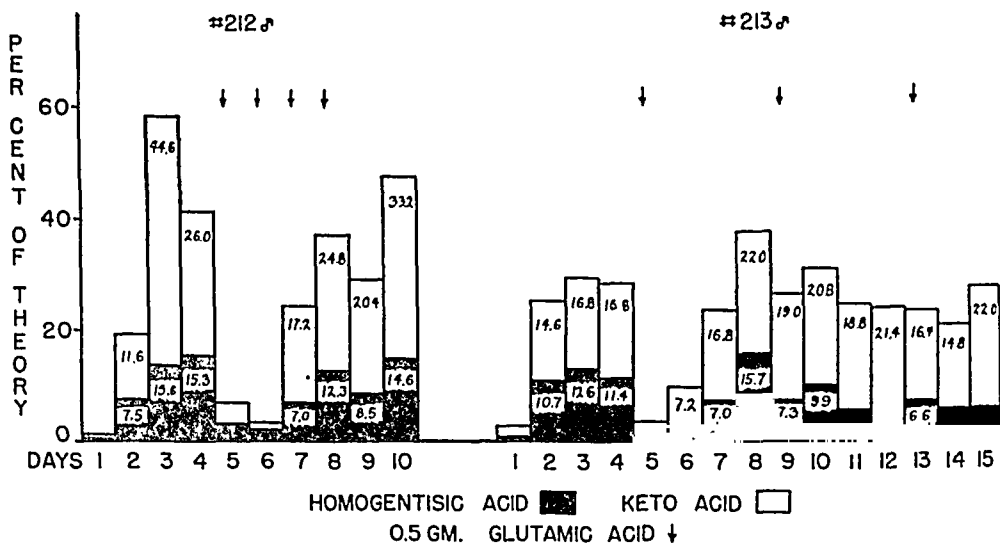


FIG. 1. Homogentisic acid (solid columns) and *p*-hydroxyphenylpyruvic acid (clear columns) excretion by guinea pigs. The administration of 0.5 gm. (3.4 mm) of glutamic acid is shown by the arrows. The per cent of theoretical yield is calculated on the basis of the amount of extra tyrosine consumed daily.

like that of ascorbic acid, but a difference is to be observed in the fact that repeated doses, unlike ascorbic acid, fail to show increasing or even continued effectiveness. Instead these experiments demonstrate a decreasing influence of the test substance. With repeated occurrence of this finding it seemed likely that the later doses were ineffective because of depleted stores of the vitamin. However, experiments to be described in the following paragraphs demonstrated that this was only a partial explanation.

The glutamic acid experiments emphasized the importance of a standardized procedure for evaluating the effect of the various test compounds. The results of preliminary experiments warranted the adoption of the

following method. The guinea pigs were removed from the stock cages where they received a diet of the basal ration plus an adequate but not excessive supply of mixed green food. When the animals were placed in metabolism cages, the daily supplement of 0.5 gm. of *L*-tyrosine was included in the deficient diet, being mixed into the upper portion of an amount of diet only slightly in excess of the amount that would be consumed in a day's time. The 24 hour urine collections were analyzed until they showed a significant excretion of the usual metabolites. By the 3rd or 4th day the excretion level was usually sufficient to justify the inclusion of the compound in question, although in a few instances this was not possible until the 5th or 6th day. A single dose of test substance was then mixed into the tyrosine-containing portion of the basal diet, so that the two compounds were eaten simultaneously and always in the first half of the 24 hour period. Since in the early experiments glutamic acid had been fed at a level of 0.5 gm. (3.4 mm), the other compounds were fed in equimolar portions. The urinary analyses were continued for 2 or 3 days. In only a few instances was the effect of the compound observed in the second 24 hour sample, and in no instance in the third. Because of this fact the decrease or change in per cent theoretical yield of metabolite in the first 24 hour period is used for comparison purposes. After an experiment of this type the guinea pigs were then returned to the stock cages and were not used again for at least 10 days.

By means of the foregoing test procedure the effect of the administration of a single dose of each of the compounds listed in Table I on the excretion of *p*-hydroxyphenylpyruvic acid was determined. Because of the relative instability of pyruvic and oxalacetic acids, alanine and aspartic acid were fed on the assumption that the animals would produce a sufficient supply of the desired keto acid. The experimental findings and the known metabolism of these two amino acids bear out this assumption.

It is evident from the data in the final column of Table I that those substances related to the oxidation cycle cause a marked decrease in the keto acid value of the urine. However, it is also evident that the compounds of similar structure produce analogous results. In fact, comparison of the two groups shows that the members of the latter are possibly even more effective in altering the course of tyrosine metabolism, producing an average change of  $-23.0$  in per cent of theoretical yield as contrasted to  $-13.8$  for those of the former. Thus it is necessary to seek some other explanation for the phenomenon observed.

At this point in the investigation it became apparent that the one feature which all of the effective compounds possessed in common is that of acidity. Should the results obtained be due solely to the acidifying action, then the feeding of these compounds with increasing amounts of alkali should cause

less and less influence on the urinary picture. Experiments of this type with succinic acid are illustrated in Fig. 2. On the one hand the free acid causes a decrease of 23 per cent of the theoretical yield of *p*-hydroxyphenylpyruvic acid, whereas in the 24 hours following feeding of the di-

TABLE I

*p*-Hydroxyphenylpyruvic Acid Excretion (Per Cent of Theoretical Yield)

The per cent of theoretical yield is calculated on the basis of the 0.5 gm. of extra *l*-tyrosine included daily in the vitamin C-free basal diet.

Test compound	Amount fed	Keto acid		
		Pre-test day	Test day	Change
	mg.	per cent	per cent	
<i>l</i> -Glutamic acid	500	28.4	19.2	-9.2
		16.8	3.2	-13.6
		26.0	3.6	-22.4
		31.0	17.5	-13.5
		16.0	17.6	+1.6
Fumaric acid	394	31.8	16.9	-14.9
Succinic "	400	40.2	1.0	-39.2
		34.2	27.2	-7.0
<i>l</i> -Malic "	455	29.6	18.3	-11.3
<i>dl</i> -Alanine	303	27.6	32.2	+4.6
	606	15.1*	0	-15.1
		32.0*	33.6	+1.6
<i>l</i> -Aspartic acid	453	16.4	0	-16.4
		49.6	25.6	-21.0
Average.....				-13.8
<i>dl</i> -Malic acid	455	22.8	0	-22.8
Glutaric "	449	26.0	0	-26.0
Tartaric "	510	17.7	15.7	-2.0
		20.3	0	-20.3
		47.7	0	-47.7
Maleic acid	394	33.2	0	-33.2
		39.2	10.2	-29.0
Tryptophane	695	25.8	22.8	-3.0
Average .....				-23.0

\* These values are excluded from the average, since 6.8 instead of 3.4 mm were used.

sodium salt there is an increased excretion of 7.5 per cent. Essentially similar results with fumaric acid are also illustrated in Fig. 2.

Repetition of these experiments with the other acids furnished overwhelming evidence of the importance of acidity of the compounds alone. The experiments of this type have been summarized in Fig. 3, from which

it is evident that as one proceeds from the more acid to the more alkaline supplement the metabolite excretion not only fails to decrease to the same extent, but, in fact, may even increase.

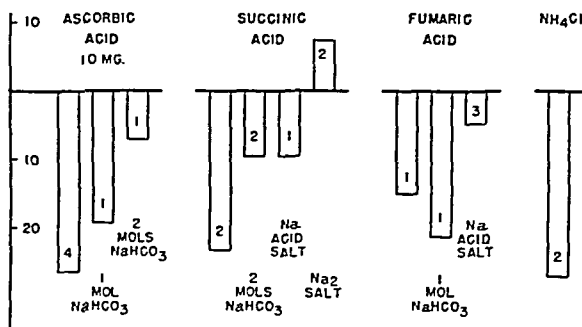


FIG. 2. The average change in per cent of theoretical yield by guinea pigs of *p*-hydroxyphenylpyruvic acid in the 24 hours following administration of 3.4 mm of test substance including the sodium bicarbonate fed with the 10 mg. of ascorbic acid. The upper ordinates indicate increased and the lower decreased excretion. The vitamin C-free basal diet included 0.5 gm. of extra *l*-tyrosine daily. The number of experiments is indicated in each column.

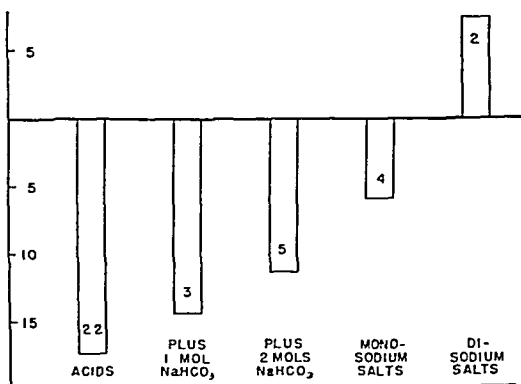


FIG. 3. The average change in per cent of theoretical yield by guinea pigs of *p*-hydroxyphenylpyruvic acid in the 24 hours following administration of 3.4 mm of test substance. The vitamin C-free basal diet included 0.5 gm. of extra *l*-tyrosine daily. The number of experiments is indicated in each column.

In view of the finding that the feeding of the dicarboxylic acids with an increasing ratio of base to acid as depicted in Fig. 3 so alters a metabolic

relationship already demonstrated to be under the specific control of the antiscorbutic vitamin (3), it becomes necessary to determine the effect of the vitamin when a highly alkaline diet mixture is consumed. This was accomplished by administering 10 mg. of ascorbic acid at the beginning of the test day. The diet consumed during the course of this day included besides the usual tyrosine supplement 3.4 or 6.8 mm of sodium bicarbonate. As shown in Fig. 2 the vitamin is less able to exert its usual influence on tyrosine metabolism when extra alkali is available. Thus strong evidence is available to support the conclusion that the effect of both acid and base observed in these experiments is closely related to if not entirely dependent upon the ascorbic acid present in the tissues of the animal. With this conclusion the failure of successive doses of glutamic acid to cause a decreased excretion of the tyrosine metabolites (Fig. 1) is in accord.

Although little doubt remained at this point of the rôle of acidity and alkalinity, additional confirmation was obtained by using the common acidifying agent, ammonium chloride, in place of the dicarboxylic acids. With an average decrease in the keto acid excretion of 27 per cent shown in Fig. 2, no further doubt remains concerning the principal nature of the phenomenon described in this paper.

#### DISCUSSION

The evidence presented forces adoption of the less interesting "acid" hypothesis rather than that of the catalytic oxidation-reduction mechanism to explain the disappearance of tyrosine metabolites from the urine when the dicarboxylic acids are administered. The tremendous amount of evidence which is accumulating from *in vitro* experiments regarding the rôle of the compounds of the Szent-Györgyi-Krebs cycles in metabolism had suggested that these systems also might be operating in conjunction with vitamin C in tyrosine metabolism. The advantages of relating this system to protein metabolism are obvious; however, the scarcity of experiments with the compounds of the cycles in intact animals is well known. On the other hand it must be emphasized that "hydrogen-carrying acids" may indeed operate in the catabolic breakdown of phenylalanine and tyrosine, and even in conjunction with ascorbic acid, although if the present *in vivo* type of experiment is used to determine such a relationship an extremely finely adjusted dietary acid-base balance is required. Experiments of this type must be reserved for the future.

With the demonstration of the acid effect on the picture of tyrosine metabolism, the next question concerns the mechanism of this effect. A clue to the mechanism is to be seen in the failure of repeated doses of glutamic acid to exert a continued decrease in the urinary keto acid value. This finding becomes understandable in the light of the work of Hawley

and associates (4, 5) on the importance of the acid-base balance to tissue storage of vitamin C. These authors have shown that the administration of ammonium chloride in amounts sufficient to produce a highly acid urine increases the excretion of the vitamin in periods of vitamin depletion as well as saturation. On this basis we may suggest that the acidifying agent in our own experiments mobilizes tissue reserves of ascorbic acid not otherwise called upon, and this newly mobilized vitamin then exerts its usual action in tyrosine metabolism. Such a possibility appears reasonable when we recall that tyrosine metabolites are absent from the urine of a guinea pig receiving 0.5 gm. of extra tyrosine after three daily doses of only 5 mg. of ascorbic acid.

Our own results emphasize not only the importance of the acid-base balance in questions of vitamin C saturation, in agreement with the above authors, but also in the consideration of the metabolic rôle of the vitamin.

#### SUMMARY

It has been found that the administration of a relatively small single dose of glutamic acid to a guinea pig receiving extra tyrosine with a vitamin C-deficient diet will cause the removal of the tyrosine metabolites from the urine. The effect observed is not permanent, for repeated doses of glutamic acid fail to produce the same effect.

By the use of other dicarboxylic acids administered with or without sodium bicarbonate or as the sodium salts and by the use of ammonium chloride the effect of glutamic acid has been demonstrated as due to the acidifying action of these compounds.

It has been further found that ascorbic acid administered when a diet containing sodium bicarbonate is being consumed fails to produce the usual vitamin C effect on tyrosine metabolism.

The relation of these findings to the importance of the acid-base balance and the question of vitamin C saturation and rôle in metabolism has been discussed.

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# FURTHER PURIFICATION OF THROMBIN: PROBABLE PURITY OF PRODUCTS\*

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In a previous publication (1) methods were given for the preparation of thrombin having a potency of 950 units per mg. of dry weight, or about 6600 units per mg. of nitrogen. With the technical improvements given below, it is now possible to isolate a fraction having a potency of 12,000 units per mg. of nitrogen. We wish to report recent solubility studies, made in an effort to determine purity. The evidence to be presented indicates that our most potent products contain less than 10 per cent of inert material. The evidence also indicates that the active material is composed of two components, which differ distinctly in solubility and probably in potency.

## *Purification of Thrombin*

*Assay of Thrombin*—In other work from this laboratory (1-3), the thrombin unit was defined as the amount of activity required to cause clotting of 1 cc. of standardized fibrinogen solution, at 28°, in 15 seconds. In much of our recent work, we have used a simplified assay technique, based upon the clotting of oxalated bovine plasma. The oxalated plasma has proved to be quite satisfactory; however, unlike the standardized fibrinogen solution, it contains no calcium ion. Because of this, and because of the presence of antithrombin, it clots more slowly. Careful comparison shows that 2.25 to 2.50 units of thrombin are required to clot 1 cc. of oxalated bovine plasma in 15 seconds.

In collection of the blood for the test, 7 parts of bovine blood are mixed with 1 part of isotonic potassium oxalate solution (1.85 per cent). The oxalated plasma, obtained by centrifugation, can be preserved as long as 2 weeks in the frozen state at -40°. In making the test, one adds 0.9 cc. of oxalated plasma to each of several tubes, together with 0.1 cc. of thrombin solution of varying dilutions. By repeated trials, and by interpolation, one determines the dilution required to cause clotting in 15 seconds. The

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tube in which clotting occurs in 15 seconds contains 2.25 thrombin units, as previously defined. After allowance is made for dilution, the titer of the original thrombin solution is readily ascertained and is expressed in units per cc.

*Special Materials*—The  $\text{Mg}(\text{OH})_2$  cream needed in this work is prepared as follows: Slowly, and with stirring, add 5 liters of concentrated  $\text{NH}_4\text{OH}$  to 20 liters of 20 per cent  $\text{MgCl}_2$ . Allow the precipitate to settle, decant, and wash the precipitate several times with water to remove ammonia. Centrifuge and suspend 500 gm. of packed  $\text{Mg}(\text{OH})_2$  in 1 liter of water.

Purified lung extract (4) was prepared as follows: To 100 gm. of fresh ground beef lung were added 100 cc. of 0.9 per cent  $\text{NaCl}$ . The mixture was allowed to stand, with occasional stirring, for 48 hours at  $5^\circ$ . Following centrifugation, a dark brown fluid was obtained and was diluted with an equal volume of 0.9 per cent  $\text{NaCl}$ . To 120 cc. of this product were added 20 cc. of  $\text{Mg}(\text{OH})_2$  cream. This adsorbs undesirable impurities. After centrifugation, 100 cc. of the supernatant fluid were mixed with 100 cc. of  $(\text{NH}_4)_2\text{SO}_4$  solution, saturated at  $5^\circ$ . The precipitate, containing the thromboplastin, was collected in the centrifuge, and was then dissolved in saline and reprecipitated with an equal volume of saturated  $(\text{NH}_4)_2\text{SO}_4$ . Following centrifugation, the final precipitate was dissolved in 15 cc. of 0.9 per cent  $\text{NaCl}$ , and was dialyzed against 0.9 per cent  $\text{NaCl}$  at  $5^\circ$  until free of  $(\text{NH}_4)_2\text{SO}_4$ . The entire procedure, including centrifugation, was carried out in the cold room. The material was stored at  $-40^\circ$ .

*Purification of Prothrombin*—Our technique has been improved from time to time (2, 1, 5) in minor particulars. The example given below represents current procedure.

At the slaughter-house 40 liters of bovine blood were allowed to flow freely, and with thorough mixing, into a series of pails containing a total of 2.4 liters of special anticoagulant ( $1.85$  per cent  $\text{K}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O} + 0.5$  per cent  $\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ ). The plasma was separated at once in a Sharples supercentrifuge (separator bowl), and was stored in the cold room at  $5^\circ$  until the following morning. At this time, 16 liters of the cold plasma were mixed in two 40 gallon earthenware crocks with 224 liters of cold distilled water (*i.e.*, 15-fold dilution of the plasma). With constant stirring, the pH was brought to 5.1 (glass electrode) by the slow addition of 2.5 liters of 1 per cent acetic acid. The precipitate of protein settled within 3 hours. The supernatant fluid was removed with a siphon and was discarded. The precipitate, with admixed liquid, measured 12.5 liters. The liquid was largely removed with the aid of a Sharples centrifuge (clarifier bowl). The precipitate now had the consistency of a thick, creamy paste. With the aid of a Waring blender it was suspended in 3 liters of special oxalated saline (0.5 per cent  $\text{K}_2\text{C}_2\text{O}_4 + 0.9$  per cent  $\text{NaCl}$ ).

The pH was brought to 6.4 by addition of 125 cc. of 0.1 N NaOH. Most of the precipitate dissolved. No effort was made to remove a small amount of flocculent material which still remained in suspension.

An adsorption procedure was then carried out. To the above solution was added 1 liter of  $\text{Mg}(\text{OH})_2$  cream. The prothrombin- $\text{Mg}(\text{OH})_2$  mixture was allowed to stand for 30 minutes at room temperature. The  $\text{Mg}(\text{OH})_2$ , with its adsorbed prothrombin, had settled, allowing much of the supernatant fluid to be eliminated by decantation. The remainder was removed by the use of a cup centrifuge. The precipitate was washed with 500 cc. of water, followed by centrifugation. It was then resuspended in 1.5 liters of water and was shaken for a 30 minute period, with  $\text{CO}_2$ , at a pressure of 2.5 atmospheres. This converts the  $\text{Mg}(\text{OH})_2$  into soluble carbonates, and at the same time releases the adsorbed prothrombin. For this step, heavy walled steel containers have been used, but rusting eventually becomes a source of annoyance. In the present experiment, the reaction was carried out in four lots, in a quart size glass Seltzer bottle. This bottle was connected to the  $\text{CO}_2$  tank with pressure tubing, and the  $\text{CO}_2$  pressure was controlled with a gage. The pressure bottle was carefully taped and suitably shielded for protection in case of breakage. During the period of the reaction the pressure bottle was rocked back and forth in a mechanical shaker. In a warm laboratory, the heat of reaction can be a source of difficulty; if the bottle becomes warmer than  $35^\circ$ , shaking should be interrupted to permit cooling, or ice should be applied.

As soon as each batch of  $\text{Mg}(\text{OH})_2$  was decomposed, 10 minutes were allowed, without shaking, and then the pressure was released slowly, thus avoiding undue foaming. The mixture was placed in an ice bath. As the foam settled, magnesium carbonates gradually crystallized out. These were removed by filtration through a Buchner funnel. The remaining electrolytes were now dialyzed away, to permit isoelectric precipitation of the prothrombin. For this purpose, the prothrombin concentrate was placed in 1 inch Visking casings (Visking Corporation, Chicago). Dialysis was carried out overnight in the cold room ( $5^\circ$ ) against 20 liters of cold distilled water. During the following day, the water was changed several times, and dialysis was again continued throughout the following night. The next morning the dialysis water was changed for the last time, and in the afternoon the white fibrinous proteins which had settled out were removed by straining through gauze.

By addition of dilute acetic acid (1 per cent), the pH was brought to pH 6.5. The proteins, which were largely inert, were removed in the cup centrifuge, and the clear fluid thus obtained was brought to pH 5.0 with acetic acid. The precipitate which formed was rich in prothrombin, and was collected in the cup centrifuge and dissolved in 40 cc. of oxalated saline

(0.1 per cent  $K_2C_2O_4$  in 0.9 per cent NaCl). The pH was brought to 7.0 by addition of 0.1 N NaOH, and the product was stored at  $-40^\circ$  until the following morning.

*Preparation and Purification of Thrombin*—The frozen prothrombin solution was brought to room temperature and was converted into thrombin by addition of 90 mg. of solid  $Ca(NO_3)_2$  and 5 cc. of the purified lung extract. After a 10 minute incubation period, the mixture was subjected to dialysis against distilled water. For this purpose the thrombin solution was placed in a 1 inch Visking sausage casing. In order to reduce the fluid volume and increase the relative surface area, a cylindrical glass plug, 1.9 cm. in diameter, weighted with mercury, and sealed at the ends, was placed within the dialysis tube. The thrombin solution thus filled the narrow space between the plug and dialysis membrane. The contrivance was then placed in a vertical glass cylinder through which cold ( $5^\circ$ ) distilled water was circulated. With this technique the salts were removed in 2 hours. As shown elsewhere (6), the use of the glass plug increases efficiency several fold.

The dialyzed thrombin solution (Solution A) then had a total volume of 43 cc., and a thrombin concentration of 21,400 units per cc. 1 per cent acetic acid was then added to bring the pH to 5.0 (glass electrode). The precipitate which forms at this stage consists largely of inert protein, to which considerable thrombin is adsorbed. Formerly this precipitate was discarded. However, we have recently found that much of the adsorbed thrombin can be removed by washing the precipitate with water; furthermore the thrombin thus set free is more potent than any previously prepared. In the present instance, the precipitate, recovered by centrifugation, was resuspended in 10 cc. of cold distilled water. On centrifugation, the supernatant fluid was found to contain 37,000 units. Nitrogen determination showed the material to have a potency of 12,000 units per mg. of N and this was almost twice as potent as any which had been prepared previously.

The precipitate, freed of a portion of the adsorbed thrombin, was now collected and resuspended in the original thrombin solution (Solution A). It then adsorbed more thrombin, and the latter was liberated by washing the precipitate in water. This cycle of adsorption and elution was repeated six more times. On the last, or eighth, elution, 25,000 units of thrombin were obtained, and the potency was 8000 units per mg. of N. From all eight elutions, a total of 220,000 units of thrombin was obtained. The remaining 700,000 units originally present in Solution A still remained partly in the latter and partly bound to the precipitate which had been employed repeatedly as an adsorbent.

During the past year many preparations of this kind have been made. Some have also been made on a much larger scale. Nineteen of the most

active fractions obtained in this manner averaged 11,492 units per mg. of N. If thrombin of this degree of purity is dried in the frozen state, it often loses some of its potency, but not always. For technical reasons it was nevertheless considered necessary to resort to drying before further study was undertaken. A supply of thrombin was collected from a large number of preparations, some of which were known to be less pure than others. The activity of the dried material used in the experiments described below was 8150 units per mg. of N, and the N content was 12.42 per cent. The material was snow-white in appearance, and very readily soluble in water.

### *Probable Purity of Products*

*Solubility Curve*—The use of solubility curves for determining the percentage composition of protein mixtures is based upon the phase rule, and has been described clearly in papers by Kunitz and Northrop (7). Solvents are so chosen that the proteins are sparingly soluble. Preliminary work with thrombin suggested the merit of using sodium sulfate solution. The latter was prepared by mixing 5 volumes of  $\text{Na}_2\text{SO}_4$ , saturated in water at  $31^\circ$ , and 4 volumes of M/15 phosphate buffer of pH 7.2. The pH of the resulting mixture, as measured with the glass electrode, was 6.45. The solvent, at  $25^\circ$ , was equilibrated with varying quantities of dried thrombin. The material which failed to pass into solution at equilibrium was separated by centrifugation at  $25^\circ$ .

The solubility curve is plotted in terms of N content and in terms of thrombin activity (Fig. 1). The upper curve of the chart shows that when small amounts of thrombin are added, all components pass completely into solution up to point A. At this point the curve changes direction for the first time. The slope changes again at points B and C. In a separate experiment, not recorded on the chart, a fourth inflection (D) occurred, and the curve became horizontal when 25 mg. of N had been added. The curve thus has four segments, indicating the presence of four protein fractions. This is in accord with preliminary electrophoretic data. The four inflection points represent the saturation points of each fraction, and the amount of each can be estimated from the slope of each segment. Thus the slope of CD is 0.068, indicating that Fraction D supplies 6.8 per cent of the total N present in the preparation. This is equivalent to stating that, beyond point C, the addition of each mg. of N permits the passage into solution of 0.068 mg. of N. Similarly the slope of BC indicates the percentage of total N in Fraction C + D and the slope of AB the percentage in Fraction B + C + D. From these data, calculations show that, on an N basis, the thrombin preparation contains 33 per cent of Fraction A, 24 per cent of Fraction B, and 36 per cent of Fraction C, and approximately 7 per cent of Fraction D.

When this work was begun, it was assumed that there would be only one

active component; however, the data point to another conclusion. The activity curve, or lower curve, of Fig. 1, which is arranged so as to permit a direct comparison with the N curve, identifies two active components with inflection points at A and C. The slope of the activity segment AC is 0.59. Component C therefore has 59 per cent of the total activity present, leaving 41 per cent of the total activity for Component A. Each mg. of N added to the solvent contributes Component A to the extent of 0.33 mg. of N, and 3362 units of activity (41 per cent of 8150 units). On this basis this component, if isolated, should have an activity of 10,086 units per mg. of N or approximately 1250 units per mg. of dry weight. In similar manner, Component C supplies 0.362 mg. of N and 4838 units.

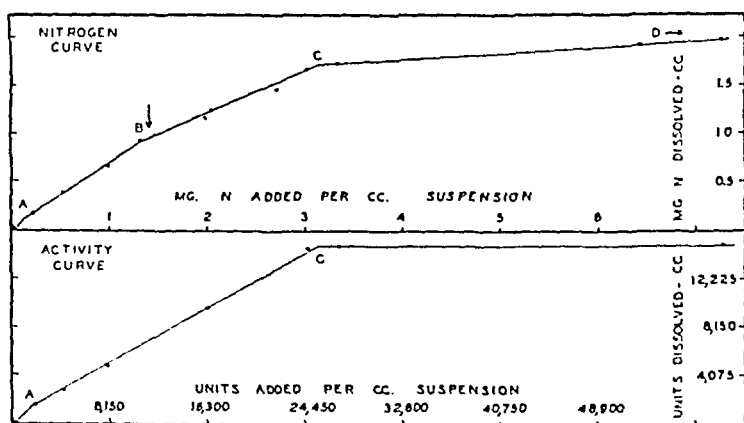


FIG. 1. Solubility curve of thrombin having a specific activity of 8150 units per mg. of nitrogen. The letters on the curves are explained in the text.

It should, therefore, have an activity of 13,365 units per mg. of N, or 1658 units per mg. of dry weight.

*Preliminary Separation of Least Soluble Thrombin*—A small quantity of thrombin was separated by equilibrating a system designated by the arrow at point B of the upper curve on Fig. 1. Theoretically this should isolate only material of pure Type A. The insoluble portion was washed once with the solvent and then dissolved in water. It assayed 10,711 units per mg. of N and therefore probably contained only a small amount of inert protein. With the hope of obtaining a solubility curve on a similar fraction, this work was followed by a similar fractionation experiment with about 400 mg. of material. The insoluble fraction was dissolved in water and dialyzed against water for the removal of sulfate and phosphate, and dried in the frozen state. This handling unexpectedly resulted in denaturation of a considerable portion of the thrombin, and

nearly half of the protein was insoluble. Consequently it was not possible to obtain the desired solubility curve. The activity of the soluble portion was in the range of that required of pure thrombin (Table I). Moreover, this activity was fairly constant over a wide range of solid to solvent ratio. In accordance with the phase rule this constitutes a criterion of purity for the range covered.

TABLE I  
*Activity of Fractionated Thrombin*

Thrombin per cc. suspension	Activity in solution*	
mg.	units per cc.	units per mg. N
3.4	2,650	11,830
11.6	10,000	13,736
27.3	17,300	11,900
40.0	24,300	11,250
55.8	32,000	11,500

\* The solvent was prepared by mixing 3.5 volumes of  $\text{Na}_2\text{SO}_4$ , saturated in water at  $31^\circ$ , and 4 volumes of M/15 phosphate buffer of pH 7.2.

#### DISCUSSION

With the present data it is not possible to reach a conclusion concerning the significance of two active thrombin components with different solubilities. The possibility of an unexplained solubility anomaly is not excluded. It is also necessary to take into account the probability of molecular changes resulting from laboratory handling before it is concluded that there are two native thrombin molecules. If, on the other hand, the new suggestion that there are two types of thrombin receives further experimental support, this will need to be taken into account in future studies on the interaction of prothrombin and thromboplastin.

In former work (1) the highest single assay for thrombin showed an activity of 950 units per mg. of dry weight or about 6900 units per mg. of N. Since that value was obtained, Astrup and Darling (8) have worked on the purification of thrombin and in one preparation had material with 1330 of their units per mg. of N. Our experiments (3) indicate that their unit is approximately 40 to 50 per cent larger than ours and, therefore, their preparations probably possessed 1930 of our units per mg. of N. More recently Milstone (9) has purified thrombin by a series of fractionations with  $(\text{NH}_4)_2\text{SO}_4$ , and reports his highest assay value to be 6000 units per mg. of N (equivalent to 36,000 of his units). None of his values is as high as those obtained in our previous work (1), and they are about 50 per cent of the average of the preparations obtained in the present study



(11,492 units per mg. of N). High purity of a product is obviously desirable in any effort to estimate the purity of a protein. Milstone (9) states that solubility data indicated inhomogeneity of his material, and no conclusion was reached from electrophoretic data. His method of preparation is quite different from ours; consequently when further purification of his product is achieved, it should be of interest to compare solubility characteristics with those described in this paper.

#### SUMMARY

Thrombin preparations previously described have been further purified. Material possessing 11,492 units per mg. of protein N can be obtained in quantity. Solubility curves indicate that these preparations contain two active components with distinctly different solubilities. Their respective potencies are approximately 10,086 and 13,365 units per mg. of N. Some preparations possessed approximately the potency required of pure thrombin. Dried products are snow-white and form water-clear solutions.

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# A MICROCOLORIMETRIC METHOD FOR MEASURING THE OXYGEN SATURATION OF BLOOD

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For purposes of measuring oxygen saturation in the arterial blood of newly born infants (1) it became necessary to develop a rapid and reasonably accurate method for small blood samples. It was further necessary to demonstrate that cutaneous blood samples could be considered identical with specimens drawn directly from an artery. The present communication describes the colorimetric method evolved for measuring blood oxygen content and capacity, and indicates its accuracy in oxygen determinations upon cutaneous blood, as compared with the results of Van Slyke-Neill manometric analyses (2) upon blood simultaneously drawn from the arteries themselves.

Drabkin and Austin (3) showed in 1935 that Beer's law of proportionality and absorption held good over an extensive range of concentrations for the pigments  $\text{HbO}_2$  and  $\text{Hb}$ , a demonstration made possible by their specially devised chamber or cell. Solutions (such as blood) too concentrated in color for analysis by usual spectrophotometric methods could be spread in this apparatus to a film 0.07 mm. in thickness, thus allowing the transmission of light for registration by the spectrophotometer. Applications of the color differences between oxyhemoglobin and reduced hemoglobin to the study of oxygen saturation of blood *in vivo* have been made by Kramer (4) and by Matthes and Gross (5). The principle is also utilized in the apparatus devised by G. A. Millikan. In such techniques a semimembranous portion of the body such as the pinna of the ear or the web between the thumb and fingers is placed between a source of light and the photoelectric cell. With suitable filters, or, as in Kramer's method, without them, the changes in color range between that of  $\text{HbO}_2$  and of  $\text{Hb}$  can thus be registered and converted to terms of oxygen saturation of the flowing blood.

Brinkman and Wildschut (6) proposed a somewhat different method for the *in vitro* determination of oxygen saturation of a small sample of cutaneous blood. This required two galvanometer readings, the first from the original sample of blood as drawn, the second from a sample of the same

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blood after its hemoglobin had been reduced by hydrosulfite solution. In an essentially similar method described by Jonxis (7) a Pulfrich step-photometer was used as the instrument of measurement and a mercury vapor lamp as the source of illumination. A blood film only 0.01 or 0.02 mm. in thickness was used, and thus only a very small sample of blood required. This was obtained from a skin puncture flowing under oil into the bottom of a pointed receptacle, whereas Brinkman and Wildschut obtained cutaneous blood as it rose in a small glass tube placed over a skin incision. In our experience this latter method of sampling was not a successful one.

The authors' method makes use of a chamber designed to fit into the micro unit of a standard Evelyn photoelectric colorimeter. A mechanical drawing of the apparatus is shown in Fig. 1.<sup>1</sup> It is similar in essentials to that devised by Drabkin and Austin (3), although less expensive, and consists of a brass cell, the cover of which (*A*) is screwed down on the bottom (*B*) to enclose two glass disks (*D*). Between the disks a thin metal shim or retaining ring (*E*) separates their surfaces by 0.006 inch, the space occupied by the blood to be examined. At *G* and *G* are holes through the brass cell wall by way of which the blood is introduced through a No. 23 hypodermic needle from the collecting syringe. The advancing edge of the blood film seems to form a sufficiently slight contact with the air displaced so that the proportions of Hb and HbO<sub>2</sub> are not altered during the process of filling. The outer surface of the cell is grooved to fit the key on the inner surface of the Evelyn micro attachment, so that the cell drops snugly into the space occupied by the bushing usually fitted there. Measurement of blood oxygen involves securing the sample, filling the cell, reading the galvanometer deflection with each of two color filters (490 and 620  $m\mu$ ), and calculating from these the oxygen content and capacity (and thus the percentage saturation) of the specimen. The details follow.

### *Method*

#### *Apparatus—*

1. Special cell for Evelyn photoelectric colorimeter, described above.
2. A 1 cc. collecting (tuberculin) syringe, fitted with a small gold pellet and a No. 23 hypodermic needle.<sup>2</sup> The pellet, which functions as an agi-

<sup>1</sup> Our first cell was made by Mr. F. J. Christensen of the Harvard Medical School Machine Shop and embodied several suggestions for which we are grateful to him. The cell is manufactured commercially by the A. L. Huntington Company, Ann Arbor, Michigan.

<sup>2</sup> In order to maintain the combined dead space in the syringes and needle as nearly constant as possible, it is advisable to use only one style of needle and to select a set of syringes the tips of which all fit the same distance into the needle.



and not allowed to drop to the bottom of the oil receptacle. If the side of the finger tip is incised and the finger inserted half an inch or so beneath the oil, the collection can easily be managed by inserting the needle beside the finger. With incisions in a larger surface, such as the heel of an infant, a conical glass vessel in the side of which a hole had been drilled was found very useful. The hole was covered by a rubber or cellophane dam, so that the vessel would hold oil to a level above this aperture. After incision the heel was immersed under the oil and the blood collected by thrusting the needle through the dam to the cut surface.

#### *Solutions—*

1. Mineral oil (sp. gr., 0.885).
2. A 50 per cent saponin solution with ammonia. 2.5 gm. of high grade saponin dissolved in 5 cc. of distilled  $H_2O$ , plus 10 drops of concentrated ammonium hydroxide. (Use no longer than 1 week.) This hemolyzes the blood and prevents it from clotting.

#### *Procedure*

Fill the dead space of the syringe (containing the gold pellet) and needle with saponin solution.

Collect in the syringe a known amount (0.3 cc.) of blood flowing freely under oil (discard the first 2 drops of blood). Shake hard until hemolysis is complete, the pellet acting as an agitator.

If oil droplets are present, remove the needle and place a rubber cap over the tip of the syringe. Centrifuge at low speed for 3 minutes, with the tip of the syringe toward the periphery. Remove the cap, and replace the cleaned and dried needle.

Discard 1 or 2 drops of blood; fill the cell with blood by inserting the needle into the hole on one side and forcing in blood until it appears at the other side.

Read in the colorimeter with Filter 490 and then with Filter 620. (Previously, determine the center settings on the colorimeter by filling the cell with saponin and water. With Filter 490, set the blank on 100; with Filter 620, set the blank cell on 85.)<sup>3</sup>

#### *Calculation*

$$\text{Total Hb} = (a \times L_{490}) + (b \times L_{620})$$

$$\text{HbO}_2 = (c \times L_{490}) - (d \times L_{620})$$

where  $L_{490} = 2 - \log G'_{490}$ ;  $L_{620} = \log 85 - \log G'_{620}$ ;  $G'_{490}$  = corrected galvanometer reading at 490  $m\mu$ ;  $G'_{620}$  = corrected galvanometer reading at 620  $m\mu$ ; and  $a = (D - C)/(AD - BC)$ ,  $b = (A - B)/(AD -$

<sup>3</sup> Since a setting of 100 could not be obtained with this filter in combination with the special cell, a blank cell setting of 85 was chosen arbitrarily.

$BC)$ ,  $c = D/(AD - BC)$ , and  $d = B/(AD - BC)$  where  $A$ ,  $B$ ,  $C$ , and  $D$  are previously determined constants obtained as follows from any normal human blood.

$$A = K_{490}^{\text{HbO}_2} = \frac{L490}{\text{Hb concentration (gm. \%)}} \text{ blood completely oxygenated}$$

$$B = K_{490}^{\text{Hb}} = \frac{L490}{\text{Hb concentration (gm. \%)}} \text{ blood completely reduced with Na}_2\text{S}_2\text{O}_4 \\ (5 \text{ mg. to 1 cc. blood})$$

$$C = K_{620}^{\text{HbO}_2} = \frac{L620}{\text{Hb concentration (gm. \%)}} \text{ oxygenated}$$

$$D = K_{620}^{\text{Hb}} = \frac{L620}{\text{Hb concentration (gm. \%)}} \text{ reduced}$$

### *Derivation of Equations Used*

By definition

$$L490 = A(\text{HbO}_2) + B(\text{Hb}) \quad (1)$$

$$L620 = C(\text{HbO}_2) + D(\text{Hb}) \quad (2)$$

From Equation 1

$$\text{Hb} = \frac{L490 - A(\text{HbO}_2)}{B} \quad (3)$$

If Equation 3 is substituted in Equation 2

$$L620 = C(\text{HbO}_2) + \frac{D}{B} (L490 - A(\text{HbO}_2))$$

Hence

$$\text{HbO}_2 = \frac{D L490 - B L620}{AD - BC}$$

or

$$\text{HbO}_2 = (c \times L490) - (d \times L620) \quad (4)$$

where  $c = D/(AD - BC)$  and  $d = B/(AD - BC)$ . Similarly

$$\text{Hb} = \frac{A L620 - C L490}{AD - BC}$$

Therefore

$$\text{Total Hb} = \text{HbO}_2 + \text{Hb} = \frac{(D - C)L490 + (A - B)L620}{AD - BC}$$

or

$$\text{Total Hb} = (a \times L490) + (b \times L620) \quad (5)$$

where  $a = (D - C)/(AD - BC)$  and  $b = (A - B)/(AD - BC)$ .

## Results

Table I presents a comparison of the results obtained by analyzing blood from identical samples with the standard Van Slyke-Neill technique (2) and with the authors' colorimetric method. The standard value 1.34 was used to convert volumes per cent of O<sub>2</sub> to gm. of hemoglobin and *vice versa*. The manometric determination necessarily involves measurements of O<sub>2</sub> in the specimen as drawn and then in a portion of it oxygenated to capacity in a tonometer; the colorimetric method does not require this extra manipu-

TABLE I

*Comparison of Results with Manometric and Colorimetric Methods on Identical Samples*

Blood No.	O <sub>2</sub> capacity		O <sub>2</sub> content		Per cent saturation		Blood No.	O <sub>2</sub> capacity		O <sub>2</sub> content		Per cent saturation	
	Mano-	Colori-	Mano-	Colori-	Mano-	Colori-		Mano-	Colori-	Mano-	Colori-	Mano-	Colori-
	metric	metric	metric	metric	metric	metric		metric	metric	metric	metric	metric	metric
	vol.	vol.	vol.	vol.				vol.	vol.	vol.	vol.		
	per	per	per	per				per	per	per	per		
	cent	cent	cent	cent				cent	cent	cent	cent		
1	24.3	24.5	15.7	15.9	65	65	20	17.1	17.1	4.2	4.4	25	26
2	24.3	24.4	24.3	24.4	100	100	21	17.1	17.1	17.1	17.1	100	100
3	24.9	24.9	8.2	8.7	33	35	22	18.9	18.8	6.9	7.5	37	40
4	24.9	24.9	24.9	24.9	100	100	23	18.9	19.0	18.9	19.2	100	101
5	19.4	19.0	9.1	9.9	47	52	24	19.4	19.3	17.7	18.1	91	94
6	17.8	18.2	6.6	6.7	37	37	25	17.3	17.3	16.5	16.6	95	96
7	17.8	18.2	17.8	18.2	100	100	26	18.5	18.4	18.0	16.7	97	91
8	21.6	21.8	4.3	5.0	20	23	27	18.8	18.6	18.1	17.1	96	92
9	21.6	21.3	21.6	21.3	100	100	28	19.3	19.3	7.8	7.9	40	41
10	18.0	18.2	2.8	3.3	16	18	29	19.9	19.6	15.7	15.3	79	78
11	18.0	18.0	12.4	13.1	69	73	30	24.1	24.1	19.5	19.3	81	80
12	19.4	19.3	9.1	9.3	47	48	31	20.5	20.6	18.8	19.2	92	93
13	19.7	18.4	18.8	18.2	95	99	32	20.6	20.8	19.5	19.2	95	95
14	20.5	20.2	20.0	19.4	98	96	33	19.0	18.9	18.1	18.0	95	95
15	18.1	17.3	12.9	12.8	71	74	34	17.9	17.7	17.0	16.6	95	94
16	18.3	17.6	14.7	14.8	80	84	35	23.4	23.8	18.8	19.8	80	83
17	20.9	19.8	20.0	19.8	96	100	36	23.6	23.3	23.6	23.3	100	100
18	19.9	19.3	19.2	18.3	96	95	37	22.4	23.7	17.7	18.0	79	76
19	20.5	20.2	20.0	19.8	98	98	38	22.3	22.9	21.6	21.8	97	95

lation. Presentation of the data in this form shows the agreement between the two methods when used for measuring oxygen content, oxygen capacity, and percentage of oxygen saturation in blood from the same source. There is no one of these categories in which results seem to agree most closely or to diverge most widely. Since the determination rests on color values, a small series of bloods with excess bilirubin was examined. Several of these (Bloods 1 to 5, Table II) were from erythroblastotic infants who were also severely anemic; the others were from infants with

TABLE II

*Results with Manometric and Colorimetric Methods on Samples from Eleven Icteric Patients*

The first five were erythroblastotic infants whose blood was both icteric and anemic.

Blood No.	O <sub>2</sub> capacity		O <sub>2</sub> content		Per cent saturation	
	Manometric	Colorimetric	Manometric	Colorimetric	Manometric	Colorimetric
	vol. per cent	vol. per cent	vol. per cent	vol. per cent		
1	10.2	11.7	2.5	3.4	25	29
2	10.2	11.0	10.2	10.6	100	96
3	8.0	7.4	6.5	5.9	75	80
4	10.5	12.9	5.3	7.9	50	61
5	10.5	12.9	10.5	12.9	100	100
6	24.8	25.1	18.6	20.0	75	80
7	20.1	20.5	12.9	14.1	64	69
8	20.1	20.0	15.8	17.0	79	85
9	20.1	20.6	20.1	20.6	100	100
10	20.6	21.0	15.7	17.2	76	82
11	20.6	20.5	20.6	20.7	100	101

TABLE III

*Comparison of Specimens Obtained Simultaneously by Cutaneous and Arterial Puncture and Examined by Different Methods*

Specimen No.	Per cent oxygen saturation	
	Cutaneous, colorimetric	Arterial, manometric
1	93	95
2	92	98
3	97	96
4	99	96
5	98	98
6	89	91
7	95	95
8	84	79
9	83	81
10	100	95
11	94	95
12	81	79
13	100	97
Average...	92.7	91.9

pronounced icterus neonatorum. It appears from Table II that the greatest errors in measurements from such specimens were with regard to oxygen capacity and that errors were largest in bloods with low



hemoglobin rather than excess bilirubin. The percentage saturation with oxygen is nevertheless reasonably accurate if determined colorimetrically even in the face of these difficulties.

In Table III are shown the results of thirteen instances in which blood was obtained simultaneously from the radial or femoral artery for manometric determination and from a finger incision for colorimetric determination. In these columns a second variable is introduced, for although Lundsgaard and Möller (8) have shown a useful agreement between cutaneous and arterial blood their exact identity is not to be expected. Nevertheless, the data show a reasonably satisfactory comparison, and one which tends to be closer in those lower ranges of oxygen saturation where measurements of this type are apt to be of most clinical utility.

#### SUMMARY

The authors have described an apparatus and method by means of which a small sample (0.3 cc. or less) of blood may be analyzed for oxygen content and capacity in the Evelyn photoelectric colorimeter. The measurement can be performed within 20 minutes. The degree of accuracy obtainable is shown by comparison with manometric determinations. It is shown by such data that the method may be applied to samples of cutaneous (capillary) blood with results which approximate those obtainable by the manometric analysis of blood secured by direct arterial puncture.

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# DETERMINATION OF CHLORIDES IN BIOLOGICAL FLUIDS BY THE USE OF ADSORPTION INDICATORS. A NEW METHOD FOR THE DETERMINATION OF THE PLASMA VOLUME OF BLOOD

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It is the purpose of this paper to present a simple and precise method for the determination of the relative volume of plasma and cells in blood. While numerous other methods for the determination of plasma volume exist, such as conductometric, refractometric, viscosimetric, and centrifugal, they are open to various objections which have been summarized by Ponder and Saslow (1). Stewart's colorimetric hemoglobin method as modified by Ponder and Saslow (2) may be accepted as a precision method, but numerous technical difficulties are encountered in its performance and it requires large quantities of blood. The photoelectric, Evans blue dye method as modified by Shohl and Hunter (3) satisfies the criteria of a precision method and has been used by us to check our own method.

The principle of the method presented depends upon the experimental fact that when whole blood is dropped slowly into a mixture of absolute alcohol and anhydrous ether (3:1), in such a manner that the cell walls are not ruptured, cell processes occur that prevent the ingress or egress of chloride ion. Therefore, when the clear, supernatant alcohol-ether extract obtained in this manner is titrated with silver nitrate, a titration figure ( $A$ ) is obtained which represents only the chloride ion in the plasma of the whole blood sample. If this titration figure ( $A$ ) is divided by ( $P$ ), the titration figure obtained by treating an equal quantity of plasma in the same manner, the fraction ( $A/P$ ) represents the relative volume of plasma in the blood. The relative volume of cells in the blood is then  $1 - (A/P)$  or  $(P - A)/P$ .

The main advantage of this method over the Evans blue dye method is that it requires nothing more elaborate than a simple titration set-up and yet gives results of the same order of accuracy. This method is especially suited for the purpose of determining the distribution of chloride ion between the cells and the plasma, as this would require only an additional whole blood chloride titration value. The procedure to be followed for the chloride ratios, the calculations involved, and the results obtained in a large

number of normal and some pathological cases will be published in subsequent papers.

### *Macroprocedure*

#### *Solutions—*

Standard NaCl, 0.1 N solution. Prepare a 0.02 N solution of NaCl by dilution of the 0.1 N solution.

Standard AgNO<sub>3</sub>, 0.1 N solution. Prepare a 0.02 N solution of the AgNO<sub>3</sub> by dilution of the 0.1 N AgNO<sub>3</sub>. Standardize the 0.02 N AgNO<sub>3</sub> solution against the standard NaCl solution in the following manner.

Pipette 1.0 ml. of the 0.02 N NaCl into a test-tube of convenient size ( $6 \times \frac{3}{4}$  inches), and add 8 ml. of alcohol-ether solution and then 2 drops of the dichlorofluorescein indicator. Titrate the samples in triplicate with the 0.02 N AgNO<sub>3</sub> using a 5.0 ml. micro burette calibrated in 0.01 ml. divisions. The end-point change is from colorless to a deep pink color appearing throughout the solution.

Alcohol-ether. A 3:1 solution by volume was made by mixing the appropriate quantities of absolute alcohol with anhydrous ether.

Dichlorofluorescein indicator, Eastman Kodak No. 373. Prepare a 0.05 per cent solution in 70 per cent alcohol.

Anticoagulant. Dissolve 6 gm. of ammonium oxalate and 4 gm. of potassium oxalate in 100 ml. of distilled water. Pipette a known volume of this solution into a 50 ml. bottle and dry in an oven at 70°. The amount of oxalate should be such that the measured blood sample contains 0.2 per cent of oxalate. The blood sample, usually 25 ml. for these runs, was obtained by venipuncture with a dry sterile syringe and was transferred to the bottle with constant stirring to prevent clotting.

Glassware used in these determinations should be washed with cleaning fluid and dried by drainage of the rinse water followed by exposure to air. Rinse all glassware with the alcohol-ether solution before use.

*Determination of Whole Blood Plasma Value (A)*—Into a 50 ml. Pyrex centrifuge tube containing about 35 ml. of alcohol-ether solution, pipette 1.0 ml. of oxalated blood with a calibrated, mark to mark type, Ostwald-Van Slyke pipette. The blood *must* be added very slowly in order to insure complete drainage of the red cells from the walls of the pipette and care must be taken to avoid dropping blood onto the walls of the tube. Stopper the tube tightly with a clean rubber stopper and stir up the red cells from the bottom of the tube by gently tapping the tube with the finger so as to avoid rupturing of the walls of the red cells. Centrifuge the tube at about 2000 R.P.M. for about 5 minutes and carefully pour off the supernatant fluid into a 125 ml. Erlenmeyer flask. Wash the precipitate twice with 5 ml. portions of alcohol-ether, stirring the precipitate with a clean, thin glass rod, recentrifuging the tube, and decanting the wash liquids into the same

flask. Add 8 to 10 drops of the dichlorofluorescein indicator and titrate with the standardized 0.02  $N$   $AgNO_3$  until a pink coloration appears throughout the solution. A fluorescent type of titration lamp and a white background will greatly enhance the sharpness of the end-point change.

*Determination of Plasma Value (P)*—Into a 50 ml. Pyrex centrifuge tube containing about 25 ml. of alcohol-ether solution pipette 0.5 ml. of oxalated plasma with a calibrated, mark to mark type, Ostwald-Van Slyke pipette. Stopper the tube with a clean rubber stopper and shake vigorously. The remainder of the procedure is exactly as given for the whole blood plasma procedure above. The titration figure is multiplied by 2 to obtain the plasma value  $P$ .

*Calculations*—Plasma volume =  $A/P$ ; cell volume =  $(P - A)/P$ .

The results obtained by this procedure were compared with those obtained by the Evans blue method with the procedure of Shohl and Hunter. 2 ml. samples of oxalated blood were used for these determinations and each sample was run in duplicate. Dye-free plasma was used as a blank and control tubes containing a known amount of dye dissolved in a given volume of plasma were run for each blood sample. An Evelyn type of photoelectric colorimeter with a 620  $m\mu$  filter (Corning No. 978, 2 mm., and Corning No. 245, 3 mm.) was used for the colorimetric readings. A typical set of results for the chloride method as compared to the dye method is given in Table I.

*Determination of Plasma Volume of Artificially Prepared Mixtures of Cells and Plasma*—As further experimental evidence that the volume of plasma can be determined by the above procedure, artificially prepared mixtures of known plasma volumes were prepared in the following manner: Draw about 100 ml. of blood, oxalated as previously described, from a healthy adult and determine the plasma volume of this blood by the chloride procedure given above. Then pipette 30.0 ml. of whole blood into a 50 ml. centrifuge tube and centrifuge at about 1000 R.P.M. for about 15 minutes. Remove exactly 5.0 ml. of the plasma in such a manner as not to disturb the red cells and transfer the 5.0 ml. of plasma to another tube which contains exactly 20.0 ml. of the whole blood. The two samples, one of which has been plasma-enriched and the other plasma-depleted by the addition or removal of a known volume of plasma, are carefully stirred with a thin glass rod and the plasma volumes determined by the chloride procedure as given above. The experimental values can then be checked against the theoretically calculated values. A set of results obtained in these runs is given in Table II. One case is also given in which the artificially prepared mixtures were determined simultaneously by the chloride and dye procedures.

*Calculations*—Since the actual amount of red cells is constant in each

case and a known amount of plasma is added or subtracted for the preparation of each mixture, the calculations are made most simply as follows:

TABLE I

*Plasma Volume Determinations by Chloride Procedure As Compared to Dye Method*

The figures represent an average of two or more results.

Sample No.	Plasma volume, chloride method	Plasma volume, dye method	Deviation
	<i>ml.</i>	<i>ml.</i>	<i>per cent</i>
1	0.661	0.660	+0.15
2	0.649	0.667	-2.70
3	0.666	0.679	-1.91
4	0.670	0.661	+0.90
5	0.660	0.650	+1.54
6	0.627	0.628	-0.16
7	0.641	0.635	+0.94
8	0.624	0.634	-1.58
9	0.714	0.700	+2.00
10	0.717	0.730	-1.78
Average.....	0.663	0.665	

TABLE II

*Plasma Volume Determinations of Artificially Prepared Mixtures of Known Plasma Volume*

Sample No.	Plasma volume, chloride method			Plasma volume, dye method		
	Experi- mental	Theoreti- cal	Deviation	Experi- mental	Theoreti- cal	Deviation
	<i>ml.</i>	<i>ml.</i>	<i>per cent</i>	<i>ml.</i>	<i>ml.</i>	<i>per cent</i>
1. Normal.....	0.741					
Plasma-depleted.....	0.670	0.688	-2.63			
Plasma-enriched.....	0.770	0.785	-1.91			
2. Normal.....	0.653					
Plasma-depleted.....	0.612	0.598	+2.34			
Plasma-enriched.....	0.736	0.722	+1.94			
3. Normal.....	0.664					
Plasma-depleted.....	0.591	0.596	-0.84			
Plasma-enriched.....	0.719	0.731	-1.64			
4. Normal.....	0.621			0.633		
Plasma-depleted.....	0.557	0.556	+0.18	0.565	0.557	+1.43
Plasma-enriched.....	0.707	0.697	+1.43	0.714	0.706	+1.13

If  $P$  represents the volume of plasma in 1 ml. of whole blood, then  $(1 - P)$  = the volume of cells per ml. of blood. For the plasma-depleted mixture,

$1 - (1 - P)/0.833 =$  plasma volume (plasma-depleted blood). For the plasma-enriched mixture,  $1 - (1 - P)/1.25 =$  plasma volume (plasma-enriched blood).

### *Microprocedure*

Because relatively large volumes of blood are required for the above procedure, it was found desirable for the purposes of routine clinical analysis to develop a microprocedure for much smaller quantities. By this procedure less than 2 ml. of oxalated blood is required to determine the plasma volume in duplicate by the chloride method as compared to a minimum of 5 ml. with the macroprocedure.

*Micro Burette*—This type of micro burette and its working principle have been described previously by Hybbinette and Benedetti-Pichler (4) and a description is also given in a text-book of microtechnique by Benedetti-Pichler (5). The mounting of the micro burette has been modified by the authors to insure greater ease of operation and its description is published here with the permission of Dr. Benedetti-Pichler.

Select a piece of capillary or thermometer tubing of uniform bore having an internal diameter of 1 mm. Calibrate the tube by means of mercury. These lengths of calibrated tubing should be about 40 cm. long. One end of the tubing is drawn out into a tip in two steps, first to a capillary of approximately 2 mm. outer diameter and then to a gradually tapering fine capillary (*c*). The tubing near the tip is then bent so as to make a vertical arm (*a*) having a length of about 3 to 4 cm. Another vertical arm (*b*) having a length of about 3 to 4 cm. is bent at the other end of the tubing in the opposite direction to the arm (*a*). The horizontal portion of the burette should be about 25 cm. The length (*a*) of the vertical arm and the length of the tip are adjusted so that, with the long part of the tube in a horizontal position, water will flow out only when the tip touches a wet surface. The proper form of the tip can be found by trial and error.

The rate of flow must be such that the meniscus in the horizontal part of the tube travels approximately 1 to 2 cm. per minute. Leave the gradually tapering, fine capillary of the tip quite long to begin with and fill the burette with water. It is sufficient to place the middle portion of the tube on a mm. scale and to hold both with the hand while these tests are being made. Short pieces of the tip are then cut off until operation of the burette and the rate of flow comply with the specifications given above. If the burette is intended for exclusive use with a certain standard solution, it is advisable to perform the adjustment of the tip with that standard solution. This will better assure the desired rate of flow in actual use.

A precision-ruled mm. scale (*e*) is mounted on the movable arm (*d*). The micro burette is mounted over the scale in a horizontal or slightly in-

clined position and is secured by means of spring clips which are held in place with copper wire. A mark is etched near the tip end so that the burette may always be mounted in the same position. A drawing of the

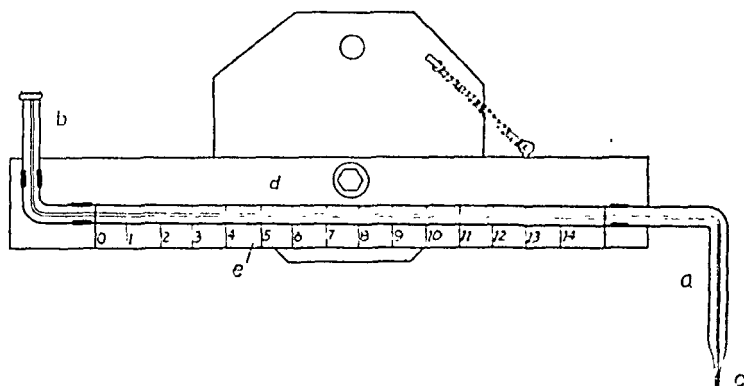


FIG. 1. Micro burette and holder. *a* is a vertical arm having a capillary tip (*c*); *b*, a vertical arm at the end of the horizontal tubing opposite *a*; *c*, a precision-ruled mm. scale, mounted on the movable arm (*d*).

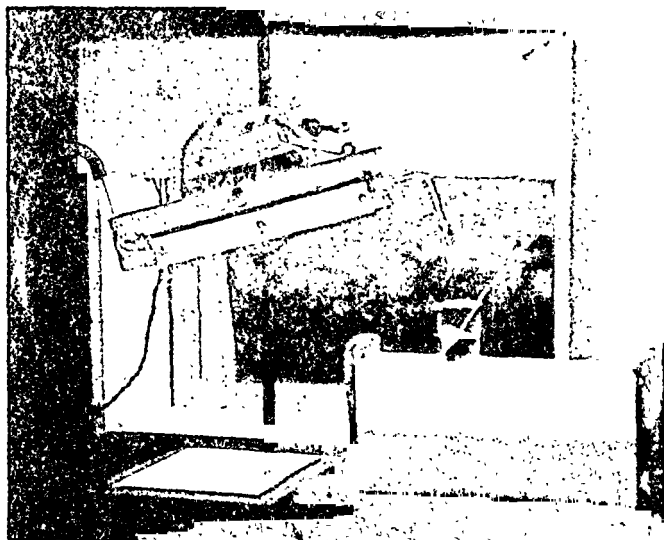


FIG. 2. Titration assembly

burette and holder is shown in Fig. 1 and a photograph of the titration assembly as actually used is shown in Fig. 2.

*Standardization of Known NaCl Solutions*—Pipette 0.2 ml. of 0.1 *N* NaCl into a 15 ml. beaker with an Ostwald-Van Slyke pipette. Add about

8 ml. of the alcohol-ether and 2 drops of the dichlorofluorescein indicator. The micro burette is filled with 0.1  $\times$  AgNO<sub>3</sub> by means of suction and the reading of the meniscus on the mm. scale is taken with the aid of a magnifying glass. The beaker containing the standard solution is placed on a stand or rack against a white background which is well lighted with a fluorescent titration lamp. The tip of the burette is kept beneath the surface of the solution until near the end-point. The titration is then continued by bobbing the tip in and out of the solution until the end-point is reached. In this manner very small amounts of liquid can be discharged from the burette. The solution is vigorously stirred during the process. The end-point change is from colorless to a deep pink color. The burette reading is taken again, and the units of the mm. scale can now be expressed in mg. of NaCl or of chloride ion.

TABLE III

*Comparison of Plasma Volume Results Obtained by Micromethod with Those Obtained by Macromethod*

The figures represent an average of two or more results.

Sample No.	Micromethod			Macromethod, plasma volume (A/P)
	Whole blood plasma value (A)	Plasma value (P)	Plasma volume (A/P)	
	<i>mm. scale units</i>	<i>mm. scale units</i>		
1	7.20	10.93	0.657	0.650
2	6.65	10.60	0.627	0.640
3	7.46	11.30	0.660	0.667
4	4.46	6.18	0.724	0.722
5	4.66	6.22	0.748	0.737
Average.....			0.683	0.683

*Microdetermination of Whole Blood Plasma Value (A)*—Into a 15 ml. Pyrex centrifuge tube containing about 7 ml. of alcohol-ether solution, pipette 0.2 ml. of oxalated blood with a calibrated Ostwald-Van Slyke pipette. Add the blood very slowly in order to insure complete drainage of the red cells from the walls of the pipette and take care to avoid dropping blood onto the walls of the tube. Stopper the tube tightly with a clean rubber stopper and stir up the red cells from the bottom of the tube by gently tapping the tube with the finger. Centrifuge the tube at about 2000 R.P.M. for about 5 minutes and carefully pour off the supernatant fluid into a 15 ml. beaker. Wash the precipitate twice with 1 ml. portions of alcohol-ether by stirring with a clean, thin glass rod. Recentrifuge the tube and decant the wash liquids into the same beaker. Add 2 drops of the



dichlorofluorescein indicator and titrate with 0.1 N  $\text{AgNO}_3$  with the micro burette in exactly the same way as given for the standard titration above.

*Microdetermination of Plasma Value (P)*—Into a 15 ml. Pyrex centrifuge tube containing about 7 ml. of alcohol-ether solution, pipette 0.2 ml. of oxalated plasma with a calibrated Ostwald-Van Slyke pipette. Stopper the tube with a clean rubber stopper and shake well. The remainder of the procedure is exactly as given for the whole blood plasma procedure above.

*Calculations*—Since the plasma volume is simply a ratio, it is sufficient to express  $A$  and  $P$  in the arbitrary mm. scale units. Plasma volume =  $A/P$ .

A set of values for the micromethod as compared with those obtained with the macroprocedure for the same bloods is given in Table III.

#### DISCUSSION

Due to war conditions it was not possible to complete the rather extensive studies which had been planned to cover such aspects of the problem as the effect of using different anticoagulants, the time of standing of the blood after withdrawal, etc., on both the plasma volume and the cell-plasma chloride ratio. The anticoagulant used in these runs was the same as that recommended by Shohl and Hunter for the Evans blue dye method. The blood samples were drawn from healthy adults among the hospital personnel and the chloride and dye determinations were performed immediately after withdrawal.

It is necessary that the alcohol-ether solutions used be as water-free as possible and that contaminations from rubber stoppers be avoided by using a set of clean rubber stoppers solely for these determinations.

Although the maximum deviation of the chloride values from the dye values reached as high as  $-2.70$  per cent, the average deviation obtained was about 1 per cent. This method was especially developed for the purpose of the determination of chloride ratios between cells and plasma; nevertheless it may prove to be a useful procedure for studies of the distribution of other biological constituents between cells and plasma.

#### SUMMARY

A new method is described for the determination of the plasma volume of the whole blood. It depends upon the experimental fact that the alcohol-ether extract of the whole blood contains *only* the plasma chlorides and not the cell chlorides. This method has been checked against the dye method and found to have an average deviation of about 1 per cent.

A new type of micro burette is described for the chloride microprocedure which makes possible the determination of the plasma volume in duplicate on 2 ml. of blood.

The accuracy of the method has been checked through the determination of the plasma volume of artificially prepared mixtures of cells and plasma.

We wish to express our appreciation to Dr. Jacob Werne, Pathologist, and Dr. Irene Garrow, Assistant Pathologist, for their most generous co-operation and permission to use the laboratories of the Flushing Hospital to perform these experiments.

We are grateful to Mr. Walter Deans, Bacteriologist, and Miss Hildegard Dzallas for the drawing of the many blood samples used in these runs and to Mr. Sidney X. Shore for the construction of the micro burette and titration lamp used in this work.

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# ESSENTIAL FACTORS FOR THE GROWTH OF THE CILIATE PROTOZOAN, COLPIDIUM CAMPYLOM

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Kidder (1) reported a successful method for the cultivation of the small ciliate protozoan *Colpidium campylo* in sterile culture media. He was able to obtain good growth (41,000 per cc.) in a medium consisting of 1 per cent yeast-Harris (The Harris Laboratories, Inc., Tuckahoe, New York), filtered in such a manner as to remove all but the smaller yeast particles, 2 per cent proteose-peptone (Difco), and 0.5 per cent dextrose (Difco). He was unable to obtain any growth in a medium consisting of only proteose-peptone and dextrose, or proteose-peptone plus dextrose and yeast-Harris which had been filtered free of all yeast particles but with the naturally occurring water-soluble components of the yeast present in the medium. He was also unable to substitute any inert particles for the yeast particles.

This work suggested that essential growth factors of unknown composition other than those present in proteose-peptone were needed by this ciliate; also that some small solid object (in this case a yeast particle) was necessary to stimulate the digestive mechanisms of the ciliate.

It is the purpose of this paper to explain the real reasons for the absence of growth in a medium free from yeast particles, and the methods and procedures used in the detection and concentration of three of the four separate growth factors found in yeast and known to be essential for the continued growth of *Colpidium campylo*.

## Material and Methods

The organisms were obtained from a sterile culture at the Arnold Biological Laboratory at Brown University. It is to be noted that this ciliate is not the same as the *Colpidium campylo* used by Hall (2) and others (see Kidder (1)).

Stock cultures were kept on a medium consisting of 1 per cent yeast-Harris, 2 per cent proteose-peptone, and 0.5 per cent dextrose prepared in the same manner as reported by Kidder (1). The organisms were grown in Pyrex, cotton-stoppered test-tubes, and the degree of growth determined by macroscopic observation of the cultures after 10 days incubation at 25°. In all cases in which growth responses were obtained, the cultures

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were carried through three successive transplants according to the procedure outlined by Lwoff (3).

#### EXPERIMENTAL

*Isolation of Growth Factors in Yeast*—The isolation and concentration of the unknown factors were carried out on Fleischmann's fresh bakers' yeast, which like yeast-Harris contains all the growth essentials for *Colpidium campylum*. The fresh yeast was first suspended in water and adjusted to pH 4.0 with phosphate buffers and then autolyzed at 37° for 3 days in the presence of chloroform, to prevent putrefaction. After autolysis the mixture was filtered (Schleicher and Schüll filter paper No. 595) free of all dead yeast cells and débris. The clear amber-colored filtrate, containing the equivalent of 1 pound of yeast per 1000 cc. of water, was used. A 10 per cent solution of this plus 2 per cent proteose-peptone and 0.5 per cent dextrose gave optimum growth.

The four factors necessary for *Colpidium campylum* and present in the yeast autolysate have been designated factor B, factor F<sub>1</sub>, factor F<sub>2</sub>, and factor IV for purposes of convenience.

Factor B is present in the non-dialyzable portion of the yeast autolysate and was separated from the autolysate by precipitation with lead acetate at pH 4.0 and 8.0.

Factors F<sub>1</sub> and F<sub>2</sub> are contained in the diffusate of the yeast autolysate and in the filtrate of the lead acetate precipitation of the autolysate. Factor F<sub>2</sub> is separated from factor F<sub>1</sub> by precipitation with phosphotungstic acid.

Factor IV has not as yet been isolated but evidence for its existence lies in the fact that yeast autolysate contributes factors which will support optimum growth in the third transplant, but the lead acetate precipitate and filtrate fractions of the yeast autolysate combined after removal of the lead by phosphoric acid and H<sub>2</sub>S will produce no growth. Factors F<sub>1</sub> and F<sub>2</sub> as contained in the lead filtrate fraction plus the non-dialyzable portion of the yeast autolysate, which is known to contain factor B, give optimum growth (Table I). These results indicate that the yeast autolysate dialysate contains another factor (factor IV) in addition to factor B, which is either destroyed by the lead precipitation, or by the H<sub>2</sub>S, or is strongly adsorbed onto the PbS.

All four factors are necessary for the growth of the organisms. In the absence of any one of the factors the organisms fail to give any demonstrable evidence of growth.

*Concentration of Factors B, F<sub>1</sub>, and F<sub>2</sub>*—Many procedures have been tested for the isolation of these growth factors from yeast and the ones found most satisfactory will be described here.

Factor B was prepared by precipitation of yeast autolysate, first with neutral lead acetate at pH 4.0, and the filtrate, after being adjusted to pH 8.0 with NaOH, reprecipitated with basic lead acetate. These two precipitates were combined and the lead removed by PbS. The filtrate after removal of the  $H_2S$  was made acid to Congo red, evaporated to a small volume *in vacuo*, and then precipitated with absolute ethyl alcohol to give a final concentration of 95 per cent alcohol. The precipitate was filtered off and discarded and the alcohol filtrate evaporated *in vacuo* to remove the alcohol and then made up to the original volume with distilled water.

After the above concentration the optimum activity for factor B was contained in 0.3 mg. per cc. of organic dry residue, which is equivalent to 20.3 mg. per cc. of organic dry residue contained in the untreated yeast autolysate.

TABLE I  
*Evidence for Presence of Fourth Factor in Yeast Autolysate*

Medium	Growth in 3rd transplant
Yeast autolysate + proteose-peptone + dextrose.. . . .	++++*
" " dialysate + proteose-peptone + dextrose. . . . .	0
" " diffusate + " + " . . . . .	0
" " dialysate + diffusate + proteose-peptone + dextrose	++++
Pb ppt. + Pb filtrate + proteose-peptone + dextrose. . . . .	0
" " + diffusate + proteose-peptone + dextrose. . . . .	0
" filtrate + dialysate + proteose-peptone + dextrose. . . . .	++++

\*++++ is indicative of optimum growth (at the 10th day).

Factor  $F_1$  was prepared from the above lead acetate filtrate. The lead was removed by adding phosphoric acid until no more precipitate was formed, filtering, and discarding the  $PbPO_4$  precipitate. The filtrate was finally treated with  $H_2S$  to remove any remaining lead. The PbS was filtered off and the filtrate made acid to Congo red and evaporated to a small volume *in vacuo*. Absolute ethyl alcohol was then added to the residue to give a final concentration of 95 per cent alcohol. The precipitate formed was filtered off and the alcohol filtrate evaporated *in vacuo* to remove the alcohol and the residue taken up in distilled water.

A saturated solution of phosphotungstic acid in 5 per cent  $H_2SO_4$  was added to the aqueous solution until no further precipitation occurred. A slight excess of the phosphotungstic acid was added and the mixture kept in the ice box for 12 hours. The filtrate (which contains factor  $F_1$ ) was then freed of excess phosphotungstate and sulfate by  $Ba(OH)_2$ . After

removal of the barium salts by filtration the filtrate was extracted for 60 hours with ethyl acetate at pH 4.0 in a continuous extractor similar in design to the one described by Hossfeld (4). The ethyl acetate was evaporated off *in vacuo* and the residue taken up in 95 per cent methyl alcohol. To this alcohol solution was added a solution of ammonium rhodanilate (prepared according to the method of Bergmann (5)) dissolved in methyl alcohol, until no more precipitate formed. The filtrate was discarded and pyridine added to the precipitate in order to remove the rhodanilate as the insoluble pyridine rhodanilate and leave factor  $F_1$  in solution. The pyridine rhodanilate was removed and the filtrate evaporated *in vacuo* to remove the methyl alcohol and pyridine and then made up to the original volume with distilled water.

After the above concentration the optimum activity for factor  $F_1$  was contained in 0.15 mg. per cc. of organic dry residue, which is equivalent to 20.3 mg. per cc. of organic dry residue contained in the untreated yeast autolysate.

The initial stages in the concentration of factor  $F_2$  are the same as those used in the concentration of factor  $F_1$ . However, the lead filtrate, after having been evaporated down to a small volume *in vacuo*, was precipitated with 95 per cent ethyl alcohol to give a final concentration of 80 per cent alcohol. The precipitate formed was removed and the alcohol in the filtrate evaporated off *in vacuo* and the residue taken up in distilled water.

The aqueous solution was then treated with a saturated solution of phosphotungstic acid in 5 per cent  $H_2SO_4$ . After complete precipitation a slight excess of reagent was added and the mixture stored in the ice box for 12 hours. The mixture was filtered and, in this case, the precipitate (which contains factor  $F_2$ ) was freed of excess phosphotungstate and sulfate by  $Ba(OH)_2$ . After removal of the barium salts the filtrate was evaporated to a gummy mass *in vacuo* and then dissolved in methyl alcohol. To this was added enough concentrated  $H_2SO_4$  to give a 5 per cent concentration. This mixture was slowly refluxed for 80 minutes. The methyl alcohol was then removed *in vacuo* and the residue taken up in distilled water. This aqueous solution was extracted with peroxide-free ether several times in a separatory funnel. The ether extracts were combined and washed first with distilled water and then with a 5 per cent solution of  $Na_2CO_3$  to remove any  $H_2SO_4$ . The ether extract was again washed with distilled water and then the extract slowly evaporated to remove the ether and the residue then made up to the original volume with distilled water.

After the above concentration the optimum activity for factor  $F_2$  was contained in 0.05 mg. per cc. of organic dry residue, which is equivalent to 20.3 mg. per cc. of organic dry residue contained in the untreated yeast autolysate.

*Miscellaneous Properties of Factors*

*Acid and Alkali Lability*—Factors  $F_1$  and  $F_2$  are completely destroyed by heating at  $120^\circ$  for 4 hours at pH 6.0. Little of their activity is destroyed by heating for 2 hours at  $120^\circ$  at pH 6.0. Factor B is stable to 2 N  $H_2SO_4$  for 6 hours at  $120^\circ$ .

Factors  $F_1$  and  $F_2$  are both completely destroyed by heating at pH 9.0 for  $2\frac{1}{2}$  hours at  $120^\circ$ , whereas factor B is stable at pH 9.0 for 6 hours at  $120^\circ$ .

*Precipitation by Heavy Metals*—Factor B is precipitated by lead and copper salts, but not by barium nor by  $HgSO_4$  in aqueous solution. Factors  $F_1$  and  $F_2$  are not precipitated by lead nor by copper in aqueous solution. Factor  $F_2$  is precipitated by  $HgCl_2$  in 80 per cent alcohol. Factor  $F_1$  is not precipitated by  $HgCl_2$  in either 80 or 90 per cent ethyl alcohol. Barium salts in alcoholic or aqueous solution do not precipitate any of the factors quantitatively. Lead was the only metal offering enough selectivity to be an aid in purification.

*Precipitation by Base-Precipitating Reagents*—Factor  $F_2$  is precipitated by phosphotungstic acid, whereas factor B and factor  $F_1$  are not. Factor  $F_1$  is precipitated by ammonium rhodanilate, whereas factor  $F_2$  is not precipitated. Factors  $F_1$  and  $F_2$  are not precipitated by picric acid.

*Adsorbents*—Factors B,  $F_1$ , and  $F_2$  are all adsorbed onto norit. None of the factors is adsorbed onto Super-Cel (Johns-Manville), kaolin, permutit, asbestos, or aluminum oxide (standardized according to Brockmann, Merck and Company). Factor B is only adsorbed onto Superfiltrol (Filtrol Corporation, Los Angeles, California) at a low pH, whereas factors  $F_1$  and  $F_2$  are readily adsorbed between pH 3.0 and 7.0.

None of the factors has been successfully eluted from norit. Factors  $F_1$  and  $F_2$  are only partly recovered from adsorption onto Superfiltrol by elution with 70 per cent ethyl alcohol at  $70^\circ$ .

*Solubility*—All three factors are insoluble in their natural states in diethyl ether, benzene, petroleum ether, chloroform, and butyl alcohol. Factors B and  $F_1$  are soluble in 95 per cent ethyl alcohol, while factor  $F_2$  is soluble in 80 per cent ethyl alcohol and only partly soluble in 95 per cent ethyl alcohol. Factor B is soluble in 80 per cent acetone. All attempts to regain the activity of factors  $F_1$  and  $F_2$  after treatment with different concentrations of acetone have been without avail. The reason for this loss of activity is at present obscure.

Factor  $F_1$  is soluble in ethyl acetate and also methyl alcohol. Factor B is insoluble in ethyl acetate. Factor  $F_2$  is insoluble in ethyl acetate in its natural form, but after acetylation the acetate is soluble in ethyl acetate and after methylation its methyl ester is soluble in diethyl ether.

*Action of Enzymes*—Neither pepsin nor trypsin destroys the activity of factor B. Pepsin and trypsin both destroy the activity of either factor



F<sub>1</sub> or F<sub>2</sub>, or both (when tested on the two together), but it has not yet been determined which of these two is inactivated or whether both are inactivated.

*Evidence of Acidic Nature*—The activity of factor B is destroyed by methylation with methyl alcohol and H<sub>2</sub>SO<sub>4</sub> plus heat. Factor F<sub>1</sub> is partially destroyed by the same treatment and factor F<sub>2</sub> is not destroyed at all under these conditions.

This experiment leads to the conclusion that factor B and possibly factor F<sub>1</sub> are predominantly acid, and that at least part of their activity is due to a carboxyl group. However, with factor F<sub>2</sub> the carboxyl group that is probably methylated is apparently not necessary for its biological activity.

*Evidence for Presence of Amino Groups*—Factors F<sub>1</sub> and F<sub>2</sub> are not destroyed by acetylation with acetic anhydride in the presence of pyridine. Factor B is destroyed by acetylation with acetic anhydride in the presence of pyridine and by benzoylation with benzoyl chloride in an alkaline medium, and it has been impossible to regenerate any of the activity of factor B by various techniques of hydrolysis tried.

Under such conditions as these benzoyl and acetyl groups are firmly attached to the nitrogen of amino groups and are resistant to hydrolysis. Thus it is indicated that there is an amino group in the compound and that it is necessary for biological activity. With factors F<sub>1</sub> and F<sub>2</sub>, if there is an amino group attacked by the acetyl radical, it is apparently one that is not necessary for the biological activity of the compound.

*Dialysis*—Factors B and IV will not dialyze through a cellophane membrane, whereas both factors F<sub>1</sub> and F<sub>2</sub> will dialyze and are found in the diffusate.

*Methods for Freeing Factor B*—Gently refluxing dried yeast-Harris in aqueous suspension for 48 hours at pH 3.0 will free about 60 per cent. Digestion of dried yeast at 60° for 5 hours with either pepsin or trypsin will free nearly 100 per cent of factor B from the yeast particles. Autoclaving the yeast in 2 N H<sub>2</sub>SO<sub>4</sub> for 6 hours frees nearly all the factor. In addition, the most effective means for bringing the factor into solution in water is by autolysis of fresh yeast cells at 37°.

### *Nitrogen or Protein Requirement*

In addition to the yeast growth factors required by this ciliate there is also what appears to be the added fastidious nitrogen or protein requirement. Growth has only been obtained in a medium of proteose-peptone plus the necessary factors in yeast. Transplantable growth does not occur in a medium of vitamin-free casein (Harris), Bacto-peptone (Difco), Bactotryptone (Difco), casein-peptone (Hoffmann-La Roche), or silk-peptone (Hoffmann-La Roche) plus the addition of the necessary factors in the yeast.

From the above results the implication is that the size of the protein degradation product is the determining factor as far as the protein requirement is concerned, particularly, since good growth is obtained with proteose-peptone, whereas with Bacto-peptone, which is a product that has been further hydrolyzed, no growth can be obtained.

Thus it appears that the organism is not able to utilize the smaller protein products (peptones and amino acids) nor the undigested proteins (casein). Such would indicate that the ciliate is unable to utilize the peptones and amino acids because of their toxicity (Dewey (6)), or because the more complex peptones and proteoses must be broken down to amino acids in a manner peculiar to the organism (Lwoff (3)).

Another possible explanation for this difference in response with proteose-peptone and Bacto-peptone is that in the preparation of the latter product some water-soluble growth essential is removed or destroyed, or some toxic material is present that is not present in the proteose-peptone.

### *Difference in Response with Different Preparations*

The kind of yeast used and its method of preparation are important factors. It was not possible to substitute an aqueous extract of dried yeast that had been filtered in such a manner as to remove all small yeast particles nor was it possible to substitute yeast extract (Difco) for the fresh autolyzed yeast. Neither was it possible to substitute an aqueous extract of either alfalfa meal (Denver Alfalfa Milling and Products Company, Lexington, Nebraska) or cerophyl (Cerophyl Laboratories, Inc., Kansas City, Missouri) for the yeast.

Table II indicates the type of results obtained with different methods of preparation of the yeast.

These results would indicate that the yeast contains all of the essential growth factors, but that at least one of these factors (factor B) is nearly insoluble in water in its natural state in the yeast. When this factor is freed from the yeast, it becomes freely soluble in water and the yeast preparation can then be filtered through a fine filter.

The possible identification of these four growth essentials here described for *Colpidium campylum* with the now recognized vitamins of the B complex that have been synthesized or isolated in a pure form is suggested. However, it is unlikely that such is the case, at least with all four of the factors, for the chemical and physical properties of these factors do not compare closely with the now recognized members of the B complex. Also, proteose-peptone is a rich source of a great many members of the B complex. Proteose-peptone (Difco) contains 16  $\gamma$  per gm. of riboflavin, 40  $\gamma$  per gm. of pantothenic acid,<sup>1</sup> and 10  $\gamma$  per gm. of thiamine.<sup>2</sup>

<sup>1</sup> Personal communication from the director of Difco Laboratories, Inc.

<sup>2</sup> As determined by the yeast fermentation method of Schultz *et al.* (7).

Some one or more of these factors may be identical with certain less well known growth factors that have been found to be necessary in animal nutrition but as yet not isolated in a pure form. However, proof of this will have to await further investigation and attempts at purification.

The author wishes to acknowledge the helpful advice and assistance given by Dr. G. W. Kidder.

TABLE II  
*Growth of Colpidium campylum with Different Yeast Preparations*

Medium	Growth in 3rd transplant
Proteose-peptone + dextrose.....	0
Yeast autolysate + ".....	0
" " + proteose-peptone + dextrose.....	++++
Yeast-Harris + proteose-peptone + dextrose.....	++++
" filtered through Seitz filter + proteose-peptone + dextrose.....	0
Yeast autolysate filtered through Seitz filter + proteose-peptone + dextrose.....	0*
Yeast-Harris filtered through Berkefeld N filter + proteose-peptone + dextrose.....	0
Yeast autolysate filtered through Berkefeld N filter + proteose-peptone + dextrose.....	++++
Yeast-Harris filtered through Jena glass bacteriological filter + proteose-peptone + dextrose.....	0
Yeast autolysate filtered through Jena glass bacteriological filter + proteose-peptone + dextrose.....	++++

\* The absence of growth in this instance is attributed to the fact that factor B is adsorbed onto the Seitz filter pad.

#### SUMMARY

1. *Colpidium campylum* requires for its growth at least four specific factors present in yeast. These factors have been designated factor B, factor F<sub>1</sub>, factor F<sub>2</sub>, and factor IV.

2. Factor B is insoluble in water in its natural state in yeast, but can be freed from the yeast cell by autolysis of the living cells, autoclaving in a strongly acid medium, or by digestion of the yeast with either pepsin or trypsin.

3. Factor B and factor IV are contained in the non-dialyzable portions of a yeast autolysate, whereas factors F<sub>1</sub> and F<sub>2</sub> are freely dialyzable through a cellophane membrane.

4. Factor B was concentrated nearly 70-fold. Factor F<sub>1</sub> was concentrated nearly 140-fold, and factor F<sub>2</sub> more than 400-fold.

5. Factor IV has not as yet been isolated.

6. The possibility of these factors being heretofore unrecognized growth essentials is indicated by the fact that their properties are different from those of other well known vitamins, and also the fact that growth cannot be obtained in a medium of only proteose-peptone or proteose-peptone plus other natural sources of the vitamins of the B complex, such as green grasses and certain types of yeast preparations, which are known to contain large amounts of the now recognized vitamins of the B complex.

7. The necessity of a protein degradation product of a specific size for this ciliate is indicated by the fact that it can utilize proteose-peptone but not Bacto-peptone.

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# INFLUENCE OF THE METHOD OF PREPARATION OF SAMPLE ON MICROBIOLOGICAL ASSAY FOR RIBOFLAVIN

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While the microbiological method for determining riboflavin has in general been a distinct improvement over preexisting methods, various workers (1-6) have pointed out serious discrepancies. In a report of collaborative work to the Association of Official Agricultural Chemists (2) high values were found for wheat flours. Recent work reported from this laboratory (6) has demonstrated the presence of substances in wheat and rice products other than riboflavin which stimulate acid production. This stimulating effect could not be corrected by adding the substances to the basal medium.

In many cases samples do not produce acid in proportion to their riboflavin content. Although different assay levels may check within 20 per cent as allowed in the original method (4), it is our opinion that, if the response is due only to riboflavin, values obtained at several assay levels should check much closer.

Strong and Carpenter (5) reported that assaying of extracts of flours and other materials after filtration at pH 4.5 gave good checks and reasonable values. A similar technique was tried in this laboratory on a variety of materials with very favorable results, which seem to clear up the difficulties encountered previously and give a method for ascertaining more nearly the correct riboflavin content of foods and feeds.

Extraction with ethyl ether, claimed by Bauernfeind *et al.* (1) to remove the stimulating effect, was also tested.

## EXPERIMENTAL

A group of representative materials was assayed for riboflavin by the original microbiological method (4). Suitable amounts of samples were autoclaved in 125 ml. of 0.1 N HCl for 15 minutes at 15 pounds pressure. The autoclaved samples were centrifuged and the supernatant liquid decanted. The residue was washed twice with 10 ml. portions of water and the washings added to the supernatant liquid. Samples containing more than 5  $\gamma$  of riboflavin per gm. of sample were autoclaved again after being centrifuged to insure complete liberation of riboflavin. The combined liquids were adjusted to pH 6.7 and assayed for riboflavin with *Lactobacillus casei*.

TABLE I  
Effect of Filtration at pH 4.5 on Riboflavin Assay

Sample	Riboflavin		Difference from original method  per cent
	Original method	Filtered, pH 4.5	
	$\gamma$ per gm.	$\gamma$ per gm.	
Citrus pulp.....	2.6	2.5	3.8
Linseed oil meal.....	3.5	3.2	8.6
Alfalfa.....	16.4	14.9	9.2
Wheat gray shorts.....	5.7	3.4	40.4
Rice bran.....	3.7	2.2	40.5
Pinto beans.....	3.5	2.0	42.9
Whole barley.....	2.3	1.3	43.5
"    oats.....	2.5	1.4	44.0
Peanut meal.....	4.9	2.2	55.1
Fish-meal.....	9.9	5.0	49.5
Soy bean oil meal.....	6.2	2.8	54.9
Corn gluten feed.....	3.0	1.3	56.7
Meat and bone scraps.....	9.8	3.9	60.2
Digester tankage.....	12.3	3.0	75.6
Whole wheat flour.....	1.8	0.9	50.0
White flour.....	0.7	0.4	42.9

TABLE II  
Riboflavin Content of Precipitate, and Recovery Values

Sample	Residue from sample equivalent	0.1 N acid pro- duced		Riboflavin apparently recovered from 0.05 $\gamma$	Apparent recovery
		Blank	Ppt.		
	mg.	ml	ml.	$\gamma$	per cent
Citrus pulp.....	48	1.05	1.05	0.085	170
Linseed oil meal.....	48	1.05	1.05	0.074	148
Alfalfa.....	12	0.60	0.62	0.074	148
Wheat gray shorts.....	48	0.95	0.95	0.114	228
Rice bran.....	48	0.60	0.60	0.106	212
Pinto beans.....	48	0.60	0.60	0.092	184
Whole barley.....	60	0.95	0.90	0.097	194
"    oats.....	60	0.95	0.95	0.124	248
Peanut meal.....	32	0.75	0.80	0.120	240
Fish-meal.....	24	0.60	1.15	0.135	270
Soy bean oil meal.....	48	0.60	1.05	0.092	184
Corn gluten feed.....	48	1.05	1.00	0.077	154
Meat and bone scraps.....	16	0.75	1.35	0.172	344
Digester tankage.....	30	0.60	1.40	0.132	264
Whole wheat flour, ether-extracted....	80	0.60	0.70	0.113	226
Cottonseed meal, ".....	32	0.60	0.80	0.148	296
Meat and bone scraps, ether-extracted	24	0.60	0.75	0.138	276
Cottonseed meal.....	32	0.60	0.95	0.130	260

TABLE III  
*Riboflavin Found Following Ether Extraction of Samples*

Sample	Original method	Ether-extracted	Filtered at pH 4.5
	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.
Whole wheat flour.....	1.8	1.93	0.91
Cottonseed meal.....	8.7	7.07	4.95
Meat and bone scraps.....	9.8	4.85	3.58

TABLE IV  
*Variation of Riboflavin Content Among Assay Levels*

Sample	Amount of sample	Riboflavin found		Deviation of successive levels
	mg.	$\gamma$ per level	$\gamma$ per gm.	per cent
Linseed oil meal	32	0.096	3.00	
	48	0.144	3.00	0.0
	10	0.049	4.91	
Fish-meal	20	0.106	5.31	+8.0
	30	0.153	5.11	-3.8
	40	0.097	2.43	
Citrus pulp	60	0.152	2.53	+4.1
	80	0.210	2.63	+4.0
	32	0.042	1.31	
Corn gluten feed	48	0.063	1.31	0.0
	40	0.049	1.23	
	60	0.076	1.23	0.0
Whole barley	80	0.097	1.21	-1.6
	40	0.054	1.35	
	60	0.078	1.30	-3.7
" oats	80	0.102	1.28	-1.5
	32	0.070	2.19	
	48	0.102	2.13	-2.7
Peanut meal	19	0.048	3.15	
	29	0.069	3.02	-4.1
	25	0.048	3.00	
Meat and bone scraps	38	0.081	3.38	+12.7
	6	0.091	15.2	
	12	0.186	15.3	+0.7
Soy bean oil meal	16	0.052	3.25	
	32	0.098	3.06	-5.8
	48	0.137	2.86	-6.5
Wheat gray shorts	16	0.055	3.44	
	32	0.107	3.34	-2.9
	32	0.064	2.00	
Pinto beans	48	0.092	1.92	-4.0
	32	0.076	2.38	
	48	0.112	2.33	-2.1



The same materials were also assayed after the cooled supernatant liquids were adjusted to pH 4.5 with a Beckman pH meter and filtered. The precipitate which formed at pH 4.5 was washed twice with a buffered solution of the same pH and the washing added to the filtrate. The solution was adjusted to pH 6.7 and assayed. The results obtained by the two procedures are given in Table I.

Differences obtained by the two methods might possibly be due to adsorption of riboflavin by the precipitate produced at pH 4.5. To investigate this possibility the precipitate was resuspended in water and the suspension adjusted to pH 6.7 and assayed for riboflavin. The effect of the precipitate in the presence of riboflavin was ascertained after known amounts of riboflavin were added to aliquots of the suspension of these precipitates. Results of these trials are given in Table II.

Samples ground in a Wiley mill through a 20 mesh sieve were dried and extracted overnight with anhydrous ethyl ether, and the riboflavin determined, with the results given in Table III. Results obtained at various assay levels of the same samples are listed in Table IV.

#### DISCUSSION

Samples prepared by filtering at pH 4.5 gave from 3.5 to 75.6 per cent less riboflavin than those prepared by the original method (4), as is shown in Table I. The differences varied with the kind of material assayed. Citrus pulp, linseed oil meal, and alfalfa give the smallest differences, while digester tankage, meat and bone scraps, and corn gluten feed give the highest.

A pH of 4.5 appears to be very near the isoelectric point of most of the proteins in solution. Thus a large precipitate forms at this point. While the proteins of samples of plant origin appear to be more completely precipitated at the above pH than animal proteins, on filtering, both types of samples yield clear filtrates.

Table II contains the results obtained when the precipitates were assayed with and without added riboflavin. From these results it is seen that the precipitates alone did not support bacterial growth, thus eliminating the possibility that the differences found in Table I are due to adsorption of riboflavin on the precipitate formed at pH 4.5. However, when 0.05  $\gamma$  of riboflavin was added to the precipitates, exaggerated responses were obtained, the apparent recovery varying from 148 to 344 per cent of the quantity actually present. Therefore, although the precipitates do not contain riboflavin, they stimulate the production of acid. This, then, can account for the higher results obtained in the original method.

Comparison of results from the ether-extracted samples with the results from the method of precipitation is given in Table III. Ether extraction does not completely remove the stimulatory materials present in the samples assayed. The ether-extracted portions give appreciably higher

results than those obtained by the method of precipitation at pH 4.5. The possibility exists that the higher results in the ether-extracted samples are due to some residual lipoidal material. Examination of material precipitated at pH 4.5 from the ether-extracted samples showed it still to contain stimulating material (Table II). Whether the substance producing the stimulation is the protein precipitated or whether the protein acts as an adsorbing or filtering agent is not known, but the method of filtration seems to be the simpler and more accurate. Work previously reported from this Laboratory (6) indicates that substances other than those extracted by ether are capable of stimulating acid production.

In Table IV are listed representative assays of samples filtered at pH 4.5, showing the results obtained at several levels, as well as differences between levels. These data show clearly that good checks were obtained among various levels. Although this is not necessarily an unquestionable criterion for measuring the validity of results, we think that it is at least a good indication of true values. In a previous paper (6) it was shown that the bacterial response to stimulatory substances decreased with increasing amounts of riboflavin. On the basis of those results, poor agreement among levels would still be obtained if such materials were present. Since good checks were obtained, the presence of non-riboflavin, stimulatory substances in the filtrate is not indicated. The method of filtration described seems to give more nearly correct results than the original method.

#### SUMMARY

1. Filtration at pH 4.5 of extracts of acid-autoclaved materials produced much lower riboflavin values than were obtained by the original microbiological method.
2. The precipitates obtained at pH 4.5 contained no riboflavin but stimulated acid production in the presence of added riboflavin.
3. Extraction of the sample with ethyl ether did not remove all the stimulating substances.
4. Agreement among assay levels was much better in samples filtered at pH 4.5 than in corresponding samples not filtered.
5. The method of filtration described seems to give more nearly correct results for riboflavin than the original method.

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# THE CHEMICAL DETERMINATION OF NICOTINIC ACID IN PLANT MATERIALS\*

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Chemical procedures for the determination of nicotinic acid, while suitable for animal tissue, give widely variable results when applied to plant materials. This is explained on the basis that the cyanogen bromide-aromatic amine reaction commonly employed in the chemical determination is not specific for nicotinic acid but for the pyridine ring with an unsubstituted  $\alpha$  position (1). Kodicek (2) and Waisman and Elvehjem (1) observed that the direct extraction of cereals with alkali or acid resulted in the extraction of non-specific chromogens (presumably pyridine compounds occurring widely in plant materials) which complicated the chemical reaction for nicotinic acid. These investigators and also Melnick, Oser, and Siegel (3) obtained values more in accord with the known nicotinic acid potency of such materials when aqueous extracts of the sample were subjected to hydrolysis. Such findings suggest that the non-specific reactions encountered in the chemical estimation of nicotinic acid in materials of plant origin might be circumvented.

Snell and Wright (4) have developed a microbiological assay method based on the production of lactic acid by the organism *Lactobacillus arabinosus*. This represents an entirely different approach than that employed by chemical procedures and offers a basis for an independent evaluation of nicotinic acid values.

The present communication deals primarily with the results of a study of the validity of various extraction and hydrolytic techniques in eliminating the non-specific reactions encountered in the chemical determination of nicotinic acid in plants. Judgments of validity are based upon (a) obtaining the lowest possible chemical values, exclusive of those resulting from errors inherent in the procedures, and (b) agreement of such values with those obtained by microbiological assay.

## EXPERIMENTAL

Modifications of the Kodicek *p*-aminoacetophenone procedure (2) were used for the chemical investigations. The chemistry of this method has been carefully investigated by Harris and Raymond (5) and Kodicek (2).

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All of the basic steps in this method were further checked in this laboratory for any errors in the procedure which might lead to erroneous nicotinic acid values. The results emphasized the importance of *correct evaluation of residual color in the blank*. On the basis of the observation of Harris and Raymond (5) that the amine reacted with pigments in urine to produce a color indistinguishable from that found with nicotinic acid, Kodicek recommended the addition of the amine to the blank determination. Methods proposed more recently by Arnold, Schreffler, and Lipsius (6) and by Bina, Thomas, and Brown (7) also include this recommendation. Melnick and Field (8) and Melnick, Oser, and Siegel (3), however, have clearly demonstrated that the above reaction does not occur in the presence of cyanogen bromide and that the amine should be omitted from the blank. Noll and Jensen (9) have further confirmed this point but in several instances it has been completely overlooked. The nature of the error introduced by

TABLE I

*Effect of Adding p-Aminoacetophenone to Blank Determination upon Nicotinic Acid Values*

Material	Amount of sample	Amine omitted	Amine added
	mg.	mg. per cent	mg. per cent
Barley	400	5.0	0.9
	1000	5.4	2.2
Wheat bran	200	24.0	5.9
	750	22.5	17.2
Alfalfa hay	400	4.7	Negative
	1000	4.4	"
Yeast	500	29.7	24.0
Beef muscle	800	17.6	14.3

adding the amine is indicated in Table I. Values obtained are low and are dependent upon the amount of material taken for analysis.

Dilute hydrochloric acid should be added to the blank solution (see the procedure below) in order to simulate the acidic nature of the reagent solutions.

*Extraction*—Water extraction of plant materials was complete within 45 minutes and extraction periods up to 2 hours did not increase nicotinic acid values. A 30 minute period occasionally failed to give complete extraction.

In the extraction of cereal grains a starchy, semisolid mass is formed. Bina, Thomas, and Brown (7) employed taka-diastase to liquefy this material. Data are presented in Table II which demonstrate that the semisolid mass may be used for analytical treatment as long as it can be pipetted accurately. When samples of about 1 gm. were used, no difficulty in handling the material was encountered.

*Hydrolysis*—Nicotinic acid derivatives are completely hydrolyzed by 1 N sodium hydroxide within 5 minutes. Acid hydrolysis usually required 30 minutes and necessitated the use of a 2 N solution.

Data in Table III demonstrate that the concentration of the hydrolytic agent, alkali or acid, does not affect the nicotinic acid values obtained for plant materials. These data suggest that trigonelline is not one of the interfering compounds found in plant extracts, as Melnick, Robinson, and Field (10) and Perlzweig, Levy, and Sarett (11) have observed that tri-

TABLE II

*Nicotinic Acid in Fractions of Cereal Extracts*

The liquid and semisolid fractions were obtained on centrifuging water-extracted samples. The solid fraction was reextracted with water before analysis.

Fraction	Wheat	Barley	Oats	Spelt
	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
Liquid.....	5.7	6.1	2.1	5.3
Semisolid.....	5.1	6.4	2.0	
Solid (reextracted).....	5.6	6.4		5.3

TABLE III

*Nicotinic Acid Values with Varying Concentrations of Acid and Alkali*

Water extracts of the sample were subjected to hydrolysis. The results are expressed in mg. per cent.

Material	Sodium hydroxide			Hydrochloric acid		
	1 N	3 N	6 N	2 N	4 N	6 N
Barley.....	6.4	6.2	5.9	8.3	8.2	8.4
Soy bean oil meal.....	4.0	4.1	4.7	9.6	9.0	9.8
Wheat.....	6.1	6.2	5.8	10.0	8.9	8.7
"    bran.....	24.2	24.5		24.9	28.5	
Alfalfa hay.....	6.1	6.2	5.9	4.2	3.9	3.5
Rape pasture.....	16.5	16.6	16.1	7.8	7.1	7.3
Muscle.....	19.2	18.3	19.6	19.1	17.3	18.3

gonelline gives increased color with increasing concentration of alkali, while being completely resistant to acid hydrolysis.

*Comparison of Various Extraction and Hydrolytic Procedures*—Extraction and hydrolysis data for a variety of plant materials and yeast are presented in Table IV. Yeast, which reacts in the same manner as animal tissue, gave values which were not affected by the various treatments so long as hydrolysis was included. Plant tissues, however, are highly sensitive to the various treatments and may be classified into two distinct types on the

basis of their responses. The seed portion of the plant, regardless of its source, always reacted in essentially the same way. The forage part of plants responded in a totally different manner than the seed and this response held for a most diverse group of plants. Values for chicory roots suggest that root products react similarly to seeds.

For seeds and their products alkaline hydrolysis of a water extract gave the lowest values of all hydrolytic procedures. Direct alkaline hydrolysis resulted in increased values, and acid hydrolysis, regardless of the mode of extraction, gave exceedingly high values. With the forage part of plants

TABLE IV

*Comparison of Extraction and Hydrolytic Procedures in Chemical Determination of Nicotinic Acid*

The results are expressed in mg. per cent.

Material	No hydro- lysis	NaOH hydrolysis of		HCl hydrolysis of	
		Water extract	Sample	Water extract	Sample
Barley.....	3.9	6.2	9.8	8.3	9.3
Soy bean oil meal .....	4.4	4.3	6.4	9.5	10.0
Wheat.....	4.5	6.0	7.6	9.2	8.1
“ bran .....	14.5	24.3	26.3	26.7	27.2
Corn.....	1.5	2.3		5.5	7.9
Cottonseed meal.....	3.3	5.0		9.9	8.5
Flour.....	1.4	2.8		5.7	
Chicory roots*.....		2.4		5.3	
Alfalfa hay .....	6.6	6.1		3.9	
Rape pasture*.....	16.1	16.4	15.1	7.4	5.8
Corn silage*.....	7.0	6.9	6.7	4.0	4.1
Blue-grass*.....	13.0	18.7		12.3	
Rumen ingesta*†.....	6.2	5.9		3.7	
Feces*†.....	3.0	3.3		1.7	
Yeast.....	18.1	33.3	31.1	30.1	

\* Values for these materials are on the dry basis.

† From a cow receiving an alfalfa hay ration.

acid hydrolysis, direct or indirect (hydrolysis of water extract), gave the lowest values, values which were much lower than those obtained when the samples were subjected to no hydrolysis at all. Both types of alkaline hydrolysis gave essentially the same value that was obtained when hydrolysis was omitted. That acid hydrolysis should give lower values than those obtained when hydrolysis is omitted is quite surprising. A possible answer to this response is suggested by the studies of Kodicek (2) in which he observed that nicotinic acid-N-diethylamide developed more color before hydrolysis than subsequently and that the color developed after hy-

hydrolysis represented the actual amount of nicotinic acid present in the compound. The possible destruction of interfering substances by the acid might offer another explanation. The response of chicory roots, the only root studied, was identical to that of the seed products.

*Agreement of Chemical and Microbiological Values*—All of the methods of extraction and hydrolysis studied above gave essentially the same values when applied to yeast and animal tissue and nicotinic acid recoveries were complete within the limit of experimental error in all instances. This led to the assumption that the lowest values obtained following hydrolysis were nearer the actual nicotinic acid content of plant materials than were the other values. To check the validity of this assumption the authors have compared these low chemical values with values obtained by using microbiological assays. The microbiological procedure of Snell and Wright (4) was employed for these studies. Application of this method to cereal products readily revealed that it gave highly variable results. When 1 N sodium hydroxide treatment replaced water extraction, however, values were obtained which were easily reproduced. These values were usually higher than those obtained by water extraction. Forage parts of the plant responded along the same lines, but values for water extracts equaled alkaline extract values in several instances. Similar observations have been noted by Teply, Strong, and Elvehjem (12) and with both acid and alkaline extraction. Values for both types of extraction are presented in Tables V and VI for comparison with the chemical values.

Nicotinic acid values for the seed portion of plants are shown in Table V for materials varying from 2 to over 25 mg. per cent in nicotinic acid content. The lowest chemical values (alkaline hydrolysis of an aqueous extract) are in excellent agreement with the highest microbiological values (alkaline extract). These findings suggest that the chemical method employed eliminated the interference of non-specific reactions and is therefore suitable for determining the nicotinic acid content of the seed and root portions of plants.

When the chlorophyll-containing portion of plants was analyzed by the method of acid hydrolysis of an aqueous extract, results were obtained which were in excellent agreement with microbiological values. The values by the chemical method represented over 50 individual determinations and were consistently obtained. Some 6 months later the same samples were again analyzed and while the response to the different hydrolytic procedures paralleled previous findings the values obtained were proportionately higher. The values for corn silage with no hydrolysis, alkaline hydrolysis, and acid hydrolysis were 10.8, 11.3, and 7.0 mg. per cent respectively compared to previous values of 7.0, 6.9, and 4.0 mg. per cent. A sample of rumen contents gave values of 9.4 mg. per cent when not hy-



drolyzed and 5.5 mg. per cent after acid hydrolysis in contrast to previous values of 6.2 and 3.7 mg. per cent. This indicates that of the methods tested acid hydrolysis of the water extract is undoubtedly the preferable

TABLE V

*Comparison of Chemical and Microbiological Values for Nicotinic Acid in Seeds and Seed Products*

Plant material	Chemical (alkaline hydrolysis of aqueous extract)	Microbiological	
		Alkaline extract (1 N NaOH)	Water extract
	mg. per cent	mg. per cent	mg. per cent
Barley.....	7.6	8.1	5.1
Corn.....	2.5	2.8	1.5
Cottonseed meal.....	5.0	4.4	2.2
Flour, low grade.....	2.6	2.6	2.4
Soy bean oil meal.....	4.0	4.0	3.6
Oats.....	1.9	2.1	
Rye.....	1.8	2.2	
Wheat.....	6.1	6.9	3.0
" bran.....	26.4	27.9	16.5
Yeast.....	30.0	28.3	29.2

TABLE VI

*Comparison of Chemical and Microbiological Values for Nicotinic Acid in Forage Materials*

Material	Chemical*		Microbiological	
	1942	1941	Alkaline extract	Water extract
	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Alfalfa hay.....	6.3	4.2	4.2	4.8
Brome ".....	8.8	3.5	3.9	2.0
Timothy hay.....	10.9-15.0	4.8		4.2
Corn silage†.....	5.5-8.3	4.0	3.4	1.9
Legume silage†.....		3.7	3.5	2.5
Rape pasture†.....		7.8	8.6	8.8
Oat pasture†.....			5.9	3.9
Rumen content†.....	5.5-9.7	3.7	3.7	3.8
Feces†.....		1.7	1.9	1.8

\* Acid hydrolysis of an aqueous extract. The same samples were used in both 1941 and 1942.

† Values for these materials are on the dry basis; others are on the air-dry basis.

procedure for forage materials; however, the data for these plant fractions (Table VI) cannot be considered as conclusive as for the seed fractions.

The authors have been unable to determine the reason for the apparent

increase in value for these samples. The recent reports of Andrews, Boyd, and Gortner (13) and of Cheldelin and Williams (14) noting the conversion of what appeared to be a precursor of nicotinic acid into nicotinic acid may offer some explanation. As pointed out by these authors, biological assays will have to be run on the material before it can be ascertained which of the values is correct and represents biologically active nicotinic acid.

On the basis of the above studies the following procedure is recommended for the determination of nicotinic acid in plant materials. The method is excellently adapted to the seed and root portions of the plant but must be used with caution, if at all, for the chlorophyll-containing forage part.

#### *Procedure for Determination of Nicotinic Acid in Plant Materials*

*Reagent Solutions*—*p*-Aminoacetophenone solution is prepared by adding 30 ml. of concentrated hydrochloric acid to 5 gm. of *p*-aminoacetophenone and making up to a volume of 100 ml. with water.

Cyanogen bromide. Distilled water saturated with bromine at 5–10° is titrated in the cold with 10 per cent potassium cyanide solution until just decolorized. A 4 per cent solution of Eastman crystalline cyanogen bromide is also suitable.

Standard nicotinic acid solution; made up in absolute ethyl alcohol to contain 100  $\gamma$  of nicotinic acid per ml.

Sodium hydroxide solution, 20 per cent.

Sodium bicarbonate solution, 5 per cent.

Dilute hydrochloric acid; 12 ml. of concentrated hydrochloric acid diluted to 100 ml. with water.

*Extraction*—A sample of plant material, usually 500 to 1500 mg., is placed in a Pyrex test-tube graduated at 15 ml. Distilled water is added and the sample is extracted on a boiling water bath for 45 minutes with occasional stirring. The sample is then cooled to room temperature, made up to volume, and centrifuged.

*Hydrolysis*—(a) 5 ml. of the aqueous extract from the seed portion of the plant are pipetted into a 200 mm. ignition tube. 1 ml. of 20 per cent sodium hydroxide is added and the extract hydrolyzed on a boiling water bath for 5 to 10 minutes. The extract is then cooled and approximately 40 ml. of ethyl alcohol are added to precipitate interfering substances. The alcoholic mixture is centrifuged and decanted into another ignition tube graduated at 50 ml. 1 ml. of 5 per cent sodium bicarbonate is added and the extract brought to pH 6 with concentrated hydrochloric acid added drop by drop from a micro burette reading to 0.01 ml. The mixture is then made up to volume with alcohol. During neutralization it is convenient to use phenolphthalein as an inside indicator and brom-thymol blue as an outside indicator. A footed glass rod is best suited for mixing the solution.

(b) 5 ml. of aqueous extract from the forage portion of the plant are pipetted into an ignition tube. 1 ml. of concentrated hydrochloric acid is added and the extract is hydrolyzed on a boiling water bath for 30 minutes. The extract is subsequently treated as under (a) with the exception that sodium hydroxide is used in bringing the solution to pH 6.

*Color Development*—Three 10 ml. portions of the neutralized extracts (representing one-fifteenth of the original sample) are pipetted into test-tubes. Tube A is used for evaluating the residual color, Tube B for measuring the color developed by the reacting nicotinic acid, and Tube C for the standard determination. 0.1 ml. of alcohol containing 10  $\gamma$  of nicotinic acid is added to Tube C. All three tubes are then placed in a water bath at 70–80° and after 10 minutes 3 ml. of cyanogen bromide are added to Tubes B and C and 5 ml. of the dilute hydrochloric acid to Tube A. After 5 more minutes the tubes are removed from the water bath and cooled to room temperature with tap water. 2 ml. of *p*-aminoacetophenone solution are added to Tubes B and C. The samples are mixed well and immediately placed in the dark. Excess light should be avoided during addition of the amine.

*Measurement of the Amount of Nicotinic Acid*—After 5 minutes and within 30 to 45 minutes following addition of the amine the color is measured in a photoelectric colorimeter with a filter having a maximum transmission of light at a wave-length of 420 m $\mu$ . Tube A is compared with a blank containing 10 ml. of 80 per cent alcohol and 5 ml. of the dilute hydrochloric acid, while Tubes B and C are compared with 10 ml. of 80 per cent alcohol plus the cyanogen bromide and amine solutions, 3 and 2 ml. respectively. Photometric density is calculated from the galvanometer readings by the formula  $L = 2 - \log G$ , where  $L$  = photometric density and  $G$  = the galvanometer reading. The micrograms of nicotinic acid in the test Solution B are calculated by the formula

$$(1) \quad \frac{(L \text{ value Tube B}) - (L \text{ value Tube A})}{(L \text{ value Tube C}) - (L \text{ value Tube B})} \times 10 = \text{micrograms in test solution}$$

The value obtained in Formula 1 and the amount of sample taken for analysis are used for calculating the mg. per cent of nicotinic acid in the plant material.

*Recovery of Nicotinic Acid and Reproducibility of Method*—Recovery values are good and indicate that none of the nicotinic acid is lost in the process of determination. Agreement of duplicate values on forty samples of seeds and seed products averaged within  $\pm 3.5$  per cent of the mean and the maximal difference was  $\pm 10$  per cent of the mean. With ten samples of forage material analyzed before the discrepancies in values were en-

countered, duplicate values agreed within  $\pm 5$  per cent of the mean, while the maximal difference was  $\pm 11$  per cent. Accuracy of the method was not increased by using two standard tubes containing different levels of nicotinic acid, as was recommended by Kodicek (2).

#### DISCUSSION

*Reliability of Nicotinic Acid Values*—The non-specificity of the cyanogen bromine-aromatic amine reaction and the varying microbiological values obtained by different methods of extraction indicate the difficulty of judging the reliability of nicotinic acid values for plant materials. If the difficulty encountered in the chemical procedures is considered to be not in measuring all the nicotinic acid present but rather in avoiding non-specific reactions (1-3) the investigator would seem limited to the consideration of the lowest chemical value he is able to obtain. If, however, it is possible that precursors of nicotinic acid are converted to nicotinic acid by the hydrolytic procedure, then the problem becomes one of ascertaining the value which represents biologically active nicotinic acid. Until biological assays are run, this value is not certain. The confusion attending a multiplicity of chemical values may be cleared up by assuming that the low values represent true nicotinic acid and nicotinic acid conjugates. On this basis the values presented in Tables V and VI are the only ones obtained by the authors which are considered acceptable. It is well to keep in mind that low values may result from errors inherent in the procedure, as is demonstrated in Table I. Naturally such values are erroneous and should be discarded.

An evaluation of the microbiological assay values also raises a problem. Extraction of the sample with acid or alkali usually results in higher values than extraction with water. The problem appears to be one of splitting some nicotinic acid conjugate which cannot be utilized by the bacteria, as suggested by Teply, Strong, and Elvehjem (12). That this is true is borne out by the fact that subsequent hydrolysis of the water extract yields values comparable to those obtained by extraction with acid or alkali. This has been noted also by Andrews, Boyd, and Gortner (13).

The excellent agreement between the most acceptable chemical values (alkaline hydrolysis of aqueous extracts from seed and root material and acid hydrolysis of forage extracts) and the most acceptable microbiological values (alkaline extraction) speaks well for the reliability of the nicotinic acid values reported in the second and third columns of Table V and in the third and fourth columns of Table VI. Agreement of values by the two methods averaged approximately  $\pm 4$  per cent of the mean for a particular sample and the maximal difference was  $\pm 10$  per cent of the mean. Such

agreement between a method based upon a chemical reaction and one dependent upon the metabolic processes of a bacterium is highly indicative that the same chemical unit is measured in both instances and gives evidence of the reliability of the values obtained by either procedure. Melnick, Oser, and Siegel (3) have found good agreement between chemical and microbiological methods applied to flour. It is interesting to note that they were able to effect a marked reduction in nicotinic acid values for flours by employing acid hydrolysis of an aqueous extract in place of direct hydrolysis of the sample. The contrary results obtained by the present method, as shown in Table IV, are probably accounted for by the different amine used or by other variations in the procedures.

Dann and Handler (15) and Hausman, Rosner, and Cannon (16) have suggested the need for complete decolorization of extracts in order to avoid non-specific reactions. The latter investigators used Lloyd's reagent in the determination of nicotinic acid in cereals and reported that when all of the pigments were not removed high values were obtained. As their procedure included direct acid hydrolysis of the sample, increased difficulty in eliminating the non-specific reactions might be expected. Agreement of chemical and microbiological assays as reported herein would indicate that non-specific reactions may be avoided without the complete removal of pigment. It is unquestionably desirable to obtain extracts with a minimum of color, even though a non-specific reaction is not involved. Extracts from seeds and seed products obtained by the proposed procedure are only slightly colored in most instances. The extracts from forages, however, contain considerable pigment which it would be desirable to eliminate and which might be the origin of some of the difficulties we have encountered with such materials.

Data presented in this paper and in the report of Teply, Strong, and Elvehjem (12) demonstrate the suitability of the Snell-Wright microbiological assay, with the precautions noted above, for all types of materials from both animal and plant source. The chemical procedure proposed for plant materials is in addition suitable for animal tissue and can be applied to urine and milk products when the specifications for hydrolysis noted by Melnick, Robinson, and Field (10) and Perlzweig, Levy, and Sarett (11) for urine and by Noll and Jensen (9) for milk are met. The chemical determination of nicotinic acid proved more convenient than the microbiological and, for feed materials other than those containing chlorophyll, gave comparable results. The discrepancies observed in chlorophyll-containing material, however, definitely limit the application of the chemical method. Some of the data obtained do give encouragement that the chemical method may eventually be adjusted to give acceptable values for forage materials in all instances.

## SUMMARY

A modification of the Kodicek procedure for determining nicotinic acid has been investigated from the view-point of application to plant materials. The use of an appropriate blank was observed to be highly important.

Concentration of the hydrolytic agent, alkali or acid, did not affect nicotinic acid values.

Plant materials may be divided into two classes on the basis of their response to various extraction and hydrolytic treatments. Alkaline hydrolysis of an aqueous extract gave the most satisfactory results for the non-chlorophyll-containing seeds and roots, while acid hydrolysis of the chlorophyll-containing part of the plant gave the lowest and most acceptable values.

Alkaline or acid treatment was necessary for the complete utilization of nicotinic acid by the bacteria employed in the microbiological assay of Snell and Wright.

Chemical and microbiological assay values for a wide variety of plant materials varying from 2 to over 25 mg. per cent of nicotinic acid were in excellent agreement. This suggests that non-specific chemical reactions have been eliminated and is indicative that reliable nicotinic acid values may be obtained by both chemical and microbiological procedures. Certain discrepancies encountered in the case of forage materials are discussed.

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## THE DISTRIBUTION OF NICOTINIC ACID IN FEEDS\*

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Recognition of the importance of nicotinic acid in the prevention and cure of pellagra has stimulated studies of its distribution in foods (12). The additional rôle of nicotinic acid in swine nutrition as observed by Chick, Macrae, Martin, and Martin (4), Davis, Freeman, and Madsen (5), and Madison, Miller, and Keith (10) renders a knowledge of its distribution in various feeds desirable.

Both chemical and microbiological methods for the determination of nicotinic acid have failed to give satisfactory results in many instances. The microbiological method of Snell and Wright (15), however, has been used for a wide variety of materials with considerable success by Teply, Strong, and Elvehjem (17) and also in this laboratory. Chemical procedures have been limited in their application largely by the non-specific reactions encountered in the assay of plant materials. Recently, however, excellent agreement between chemical and microbiological values for a wide variety of materials from plant sources was obtained by Hale, Davis, and Baldwin (6) when the chemical procedure included alkaline hydrolysis of an aqueous extract. Non-specific reactions were sometimes encountered with forage materials but with seeds, seed products, and roots all non-specific reactions were apparently eliminated. Melnick, Oser, and Siegel (13) have reported success in the chemical estimation of nicotinic acid in flours and breads with good agreement of chemical and microbiological values. Noll and Jensen (14) and Bailey, Dann, Satterfield, and Grinnells (3) have adapted the chemical method to milk products and, in general, chemical methods are well suited for the assay of animal tissue. Thus it is noted that chemical methods have been adapted with success to all types of animal and plant materials with the exception of forages, in which erratic results have been obtained. The immediate need for nicotinic acid values of feedingstuffs as an aid to compounding animal rations has therefore been expanded to include determinations on a wide variety of feed materials. The results of the nicotinic acid analyses are reported in this communication.

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TABLE I  
Nicotinic Acid Content of Feedingstuffs

Feedingstuff	Nicotinic acid	Values from literature	
Cereals			
	<i>mg. per cent</i>	<i>mg. per cent</i>	
Barley.....	6.0, 6.8, 7.6, 8.1*	4.7*	Tepley <i>et al.</i> (17)
Corn, yellow.....	1.7, 2.5, 2.8*	1.6-2.6*	" " "
		1.3	Kringstad and Naess (9)
		1.2-1.6	Aykroyd and Swaminathan (2)
		2.6*	Snell and Wright (15)
" germ.....	3.1		
" gluten feed....	10.4		
" " meal ..	3.0		
Hegari.....	6.8		
Milo.....	8.6		
Kafir.....	4.0		
Oats.....	1.8, 2.0, 2.1*	1.1, * 1.6*	Tepley <i>et al.</i> (17)
		1.0, 1.1	Aykroyd and Swaminathan (2)
" hulled.....	2.6		
Rice, polished.....	1.2	0.9*	Tepley <i>et al.</i> (17)
Rye.....	1.7, 2.2*	0.9-1.3*	" " "
		1.3*	Ihde and Schuette (7)
		1.3	Kringstad and Naess (9)
Spelt .....	4.9, 5.7		
Wheat.....	6.0, 7.0, 9.6, 6.9*	4.7, 5.3	Aykroyd and Swaminathan (2)
		6.7-5.4*	Snell and Wright (15)
		5.0-7.0*	Tepley <i>et al.</i> (17)
" bran.....	24.2, 27.4, 27.9*	25.7, * 28.7, *	" " "
	29.2, 40.9	40.0*	
" germ.....	5.2	4.2	Kringstad and Naess (9)
		3.4, * 4.7*	Tepley <i>et al.</i> (17)
		9.1	Aykroyd and Swaminathan (2)
" standard			
middlings ..	10.7, 6.1	10.6, * 13.0*	Tepley <i>et al.</i> (17)
" flour			
middlings..	10.3	9.2*	" " "
" white shorts..	8.9		
" red dog flour..	4.6		
" flour, low			
grade.....	2.6, 2.6*		
Miscellaneous feeds			
Cottonseed meal.....	4.1, 5.0, 4.4*		
Linseed meal.....	5.5	4.3*	Tepley <i>et al.</i> (17)
Palm kernel oil meal.	4.4		

TABLE I—*Concluded*

Feedingstuff	Nicotinic acid	Values from literature	
Miscellaneous feeds—Concluded			
	mg. per cent	mg. per cent	
Soy beans.....	4.0	4.8	Swaminathan (16)
“ bean oil meal, toasted.....	3.6, 4.0, 4.0*		
Soy bean oil meal, expeller.....	4.0		
Soy bean oil meal, raw solvent.....	3.8		
Beet pulp.....	2.6		
Chicory root.....	2.3		
Beet molasses.....	5.0, 4.7*		
Cane “.....	4.4, 5.0*		
Meat and bone scraps.....	4.6, 8.2, 7.1		
Tankage.....	6.7		
Fish-meal, white.....	6.9, 9.0		
Beef muscle.....	4.4	6.4	McIntire <i>et al.</i> (11)
“ liver.....	21.2	15.1–22.7	“ “ “
Bakers' yeast.....	32.8, 30.0, 28.9,* 54.3	40.0–50.0*	Tepley <i>et al.</i> (17)
Milk.....		0.08–2.8	Bailey <i>et al.</i> (3)
		0.08*	Snell and Wright (15)
		0.08*	Tepley <i>et al.</i> (17)
Skim milk.....		0.06–0.09	Noll and Jensen (14)
“ “ powder...	1.4,* 1.6*	1.4–2.8	“ “ “
		0.9*	Tepley <i>et al.</i> (17)
Buttermilk powder..	1.3,* 2.1*		
Roughages			
Alfalfa hay.....	3.6, 4.2, 4.2*	3.9*	Tepley <i>et al.</i> (17)
Brome “.....	3.5, 4.0*		
Clover “.....	3.5, 4.8		
Timothy“.....	4.8, 4.2*	2.3*	Tepley <i>et al.</i> (17)
Alfalfa leaf meal....	5.3		
	Nicotinic acid as fed	Dry basis	
	mg. per 100 gm.	mg. per 100 gm.	
Corn silage.....	1.5, 1.4, 1.2*	4.7, 4.0, 3.4*	
Legume “.....	1.0, 0.94*	3.7, 3.6*	
Alfalfa pasture....	1.8	9.3	
Clover “.....	1.6, 2.4*	9.7, 15.7*	
Rape “.....	1.0, 1.2,* 2.0	7.8, 8.6*	
Oat “.....	0.82*	5.9*	
Blue-grass pasture...	1.4*	6.6*	

\* Microbiological assay values.

## EXPERIMENTAL

Most of the original values reported in this paper were obtained by the chemical procedure outlined by Hale, Davis, and Baldwin (6). In several instances microbiological values obtained by the method of Snell and Wright (15), modified to include alkaline treatment, are also presented. All succulent materials assayed were oven-dried prior to analysis. The values obtained for a wide variety of feeds are presented in Table I. A number of selected values from the literature are presented for comparison. The aim in selecting values was to present those most representative of the literature which were obtained on similar samples by the use of reliable procedures. Both chemical and microbiological values are presented.

Several cereals are highly deficient in nicotinic acid. Oats, rye, corn, and polished rice all contained 2 mg. per cent or less and it is in those regions where rice or corn forms the staple food that pellagra is most common. It is also of interest that Davis, Freeman, and Madsen (5) observed pigs receiving corn or oats were more susceptible to necrotic enteritis than those fed wheat or barley. Barley, wheat, spelt, milo, hegari, and kafir usually contained about 5 to 8 mg. per cent of nicotinic acid. Wheat bran was an excellent source of nicotinic acid, with values from 24.2 to 40.9 mg. per cent. The nicotinic acid content of various wheat by-products would seem to be primarily dependent upon the amount of bran included in them with most of the middling products containing nearly 10 mg. per cent. Bran from other cereals appears equally rich, as Arnold, Schreffler, and Lipsius (1) have reported a value of 29 mg. per cent for rice bran. Corn gluten feed, a mixture of corn gluten meal and corn bran, contained 10.4 mg. per cent of nicotinic acid. Neither wheat nor corn germ is a particularly good source of nicotinic acid, as values of 5.2 and 3.1 mg. per cent respectively were obtained. The value for wheat germ is comparable to most values from the literature. Gluten products are low in nicotinic acid. A value of 3.0 mg. per cent was found for corn gluten meal and Teply, Strong, and Elvehjem (17) reported a value of 2.5 mg. per cent for wheat gluten.

Various protein supplements such as cottonseed meal, linseed meal, palm kernel meal, and soy bean oil meal contain from 3.6 to 5.5 mg. per cent. Protein supplements from animal sources are not as rich in nicotinic acid as might be expected. Meat and bone scraps and tankage contained from 4.6 to 8.2 mg. per cent and fish-meal from 6.9 to 9.0 mg. per cent. Milk and milk products are very low in nicotinic acid, with most of the values between 0.8 and 2.0 mg. per cent on the dry weight basis. Beet pulp, chicory roots, and beet and cane molasses are also quite low in nicotinic acid. Liver and yeast are excellent sources.

Dry roughages such as alfalfa, clover, brome, and timothy hay are not especially good sources and usually contain approximately 4 mg. per cent

of nicotinic acid. A similar value was found for both corn and legume silage on the dry basis. Pastures are a better source of nicotinic acid than are dry roughages, with values sometimes reaching 15 mg. per cent on the dry weight basis. Legume pastures ranged from 9.3 to 15.4 mg. per cent, while non-legume pastures varied from 5.9 to 8.6 per cent. Fresh pasture samples varied from 0.82 to 2.4 mg. per cent.

#### DISCUSSION

A careful survey of Table I readily shows that the nicotinic acid values reported for most all of the materials are well substantiated by both chemical and microbiological assays and by reports from various laboratories. This gives evidence of the acceptability of both the values reported and the various methods employed in their determination.

It probably should be noted that Kodicek (8) and Thomas, Bina, and Brown (18) have reported a number of values for cereals and other materials which are lower than those presented in Table I. Those values were omitted, however, because the chemical procedures used included addition of the amine to the residual color blank. Such a procedure is known to yield erroneously low values (13, 6). Values for milk products higher than those presented in Table I have been reported in the literature but the methods employed were questionable regarding their adaptability to milk and milk products. The consistently low values obtained by both chemical and microbiological assays indicate that the lower values recently reported are more representative of the nicotinic acid content of milk.

Variations in the nicotinic acid content of various pasture plants are contrasted with the apparent uniform values obtained for hays and silages from all plant sources. The lower values obtained for the hays and silages, usually about 4 mg. per cent, suggest that nicotinic acid values may vary with the stage of maturity and that the variations obtained with pasture may be due to this rather than to differences in the plant source.

#### SUMMARY

Nicotinic acid values for a wide variety of feedingstuffs are reported. Oats, rye, corn, polished rice, low grade flour, beet pulp, milk, and milk products are very deficient in nicotinic acid.

Legume and non-legume hays and silages, germ and gluten products of corn and wheat, red dog flour, high protein supplements from plant sources, soy beans, and molasses ranged from about 3 to 5 mg. per cent of nicotinic acid.

Wheat, barley, spelt, grain sorghums, tankage, meat and bone scraps, fish-meal, and non-legume pastures varied from approximately 5 to 9 mg. per cent.

Wheat middlings and corn gluten feed usually contained about 10 mg. per cent of nicotinic acid and legume pastures varied from approximately 10 to 15 mg. per cent.

Wheat bran (probably all cereal brans), yeast, and liver are excellent sources.

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# A STUDY OF THE AVAILABILITY OF MESOLANTHIONINE FOR THE PROMOTION OF GROWTH WHEN ADDED TO A CYSTINE-DEFICIENT DIET

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Of interest in connection with the recent isolation of the thio ether amino acid, mesolanthionine, from acid hydrolysates of alkali-treated proteins (1, 2) is the question of its physiological availability. The fact that the two related sulfur-containing amino acids, methionine and cystine, are readily utilized in the nutrition of animals suggested that lanthionine might also be utilized. Lanthionine, like methionine, is a thio ether amino acid, and it apparently originates from cystine in proteins by the action of dilute alkaline solutions. The mechanism of the formation of lanthionine from wool has been recently hypothesized by Nicolet and Shinn (3). Du Vigneaud, Brown, and Chandler (4) have shown that the unsymmetrical thio ether amino acid, *l*-S-( $\beta$ -amino- $\beta$ -carboxyethyl)homocysteine, can serve in lieu of cystine in the diet of rats for the support of growth. Evidence was later obtained (5) showing that rat liver tissue is capable of cleaving this thio ether amino acid with the formation of *l*-cystine. Brand and associates (6) found some evidence that carboxymethylcysteine is cleaved by cystinuric patients. It seemed possible that mesolanthionine also might be cleaved in the animal body to form cysteine, and thereby be capable of being utilized in the diet in lieu of cystine.

The question arises whether the internally compensated mesolanthionine can be cleaved by digestive enzymes. As far as we are aware, the only instance in which a mesoamino acid has been shown to be cleaved in the animal body is that in the case of mesocystine (7). Cleavage of 1 molecule of mesolanthionine could yield either 1 molecule of *l*-cysteine or 1 molecule of *d*-cysteine, depending on which side of the sulfur in the mesolanthionine molecule the cleavage took place. Since it has been shown (8) that *d*-cystine is not utilized for growth, failure of animals to grow on mesolanthionine would, therefore, indicate either that cleavage did not occur or that it occurred in such a way as to yield *d*-cysteine.

## EXPERIMENTAL

Five lots of albino rats weighing 45 to 50 gm. each were used in this study. All the rats in each individual lot were from the same litter and

were equally distributed as to sex. The basal diet had the following percentage composition: casein 6.0, dextrin 37.0, sucrose 15.0, salt mixture (Osborne and Mendel (9)) 4, agar 2.0, lard 19, cod liver oil 5, and milk vitamin concentrate 12.0. The diet was fed *ad libitum* and a record of the

TABLE I  
*Food Consumption*

Lot No.	Rat No.	Experimental period	Supplement to basal diet	Average daily food consumption
		<i>days</i>	<i>per cent</i>	<i>gm.</i>
284	1997 ♂		0.30 cystine	6.5
262	1757 ♂	9	No supplement	4.0
		31	0.30 cystine	5.7
260	1740 ♀	6	No supplement	4.1
		25	0.37 methionine	6.2
		9	No supplement	4.6
262	1759 ♂	9	" "	5.2
		31	1.0 mesolanthionine	3.7
267	1800 ♀	15	1.0 "	4.7
267	1801 ♂	15	1.0 "	5.0
260	1738 ♂	39	No supplement	4.4
260	1737 ♀	6	" "	6.1
		11	0.52 mesolanthionine	4.2
		14	0.37 methionine	7.4
		10	No supplement	4.6
260	1739 ♂	6	" "	5.1
		11	0.52 mesolanthionine	4.3
		14	0.3 cystine	5.0
		19	No supplement	4.0
260	1736 ♂	6	" "	5.3
		11	0.3 cystine	7.6
		14	0.52 mesolanthionine	5.4
		19	No supplement	4.2
284	1996 ♂	14	0.3 cystine	6.7
		25	0.52 mesolanthionine	4.6
		13	1.0 "	3.4
267	1799 ♀	15	1.0 "	4.6
261	1748 ♀	44	No supplement	3.3

This table includes the record of only those rats whose growth curves are shown in Figs. 1 and 2.

food intake is given in Table I. The rats fed the basal diet alone invariably lost weight rapidly and in several cases died within 40 to 45 days. For comparison with the behavior of the rats given mesolanthionine, *l*-cystine and *dl*-methionine were also used. Addition of 0.3 gm. of cystine or 0.37 gm. of methionine to 100 gm. of the basal diet caused immediate resumption

of growth. In most cases 0.52 gm. of mesolanthionine was added. These amounts of the supplements represent sulfur equivalents. Increasing the amount of mesolanthionine to 1.0 gm. gave no better results than when fed at the lower level. The mesolanthionine used was prepared from human hair as previously described (2). The amino acid supplements were incorporated in the basal diet.

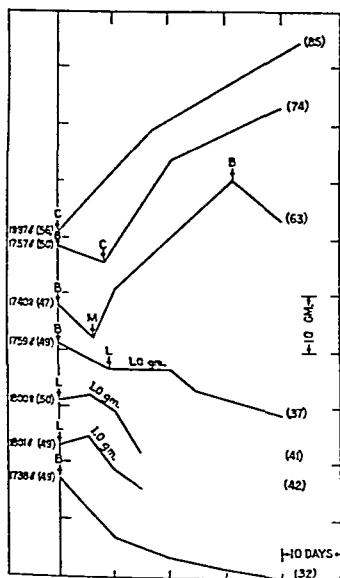


FIG. 1

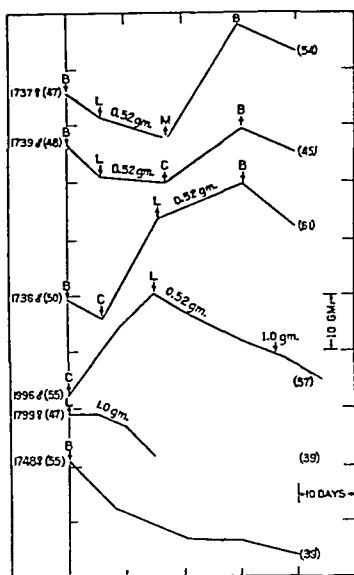


FIG. 2

FIGS. 1 AND 2. Growth curves of rats receiving the basal deficient diet (B) alone or supplemented with 0.3 per cent of *l*-cystine (C), 0.37 per cent of *dl*-methionine (M), or with mesolanthionine (L) in amounts indicated on the curves. The introduction of each diet is indicated by the symbols over the downward arrows. The identification number and sex of rats are given on the extreme left and their initial and final weights in parentheses.

All of the rats of Lot 260 (eight rats), Lot 261 (eight rats), and Lot 262 (six rats) were fed the basal diet at the start with no supplement for 6 to 9 days. Two rats in each of these lots were continued on the basal diet throughout the feeding period to serve as controls. In Lots 260 and 261 the effect of the supplements was studied through successive periods on the same animal. In Lot 262, following the fore period on the basal diet, each animal was fed one of the supplements throughout the experiment. Lot 267 (three rats) received mesolanthionine from the start without the pre-



liminary feeding of the basal diet alone. Of the four rats of Lot 284 two were fed at the beginning the basal diet plus cystine and the other two rats were fed the basal diet plus methionine. After 14 days one rat from each of the two pairs in Lot 284 was changed to the basal diet plus mesolanthionine. The other two were allowed to continue on the cystine and the methionine diets. The growth curves of representative animals from each group are given in Figs. 1 and 2, and they are typical of the others which are omitted in order to save space.

#### DISCUSSION

Five of the six rats of Lots 260, 261, and 262 that received mesolanthionine, after having first been fed the basal diet alone for a period, continued to decline in weight and they did little or no better than when they were getting the basal diet alone.

The three rats of Lot 267 fed mesolanthionine from the start, without preliminary feeding of the basal diet alone, increased slightly in weight during the first 6 days, but thereafter declined in weight at a rate comparable with the rats receiving the basal diet alone.

In striking contrast to the rats of Lots 260, 261, and 262 that failed to respond to mesolanthionine after having declined on the basal diet alone, those that similarly received cystine (six rats) and methionine (six rats) responded at once and made rapid gains, as did also those that were given cystine and methionine after they had failed to respond when given mesolanthionine (Rats 1737 and 1739).

Of all of the rats used in this study only two failed to decline in weight promptly when mesolanthionine was used as the amino acid supplement. Rats 1735 and 1736 had responded immediately to methionine and cystine, respectively, but when these amino acids were replaced by mesolanthionine the expected decline did not occur, although the rate of gain in weight was definitely retarded. Since the continued growth might possibly be due to some carry-over effect of the methionine and cystine, a new lot of four rats was started. Rats 1994 and 1995 were given methionine and Rats 1996 and 1997 were given cystine at the start. After 14 days of good growth the diets of Rats 1995 and 1996 were supplemented with 0.52 gm. of mesolanthionine instead of the methionine and cystine. The diets of the other two rats were not changed. The two rats given mesolanthionine started to lose weight at once and declined rapidly, while those receiving methionine and cystine continued to grow at a very good rate.

The results of these experiments show quite conclusively that mesolanthionine cannot serve in lieu of cystine in the diet of the rat for the support of growth.

It is of interest to note that Steinberg (10) in a study of the availability of

the sulfur of different compounds for the growth of *Aspergillus niger* found that the sulfur of mesolanthionine was utilized to a very low degree as compared with that of cystine and methionine.

Inasmuch as rats are unable to utilize *d*-cystine for growth, their failure to grow on the basal diet supplemented with mesolanthionine indicates that either they were unable to cleave the thio ether amino acid or that cleavage occurred with the formation of the unutilizable *d*-cysteine instead of *l*-cysteine. It seems possible, however, that *l*-lanthionine can be utilized for growth. In order to obtain information on this point a quantity of *dl*-lanthionine (11) is being prepared for feeding experiments.

#### SUMMARY

Mesolanthionine cannot serve in lieu of cystine in the diet of the rat for the support of growth.

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# OCCURRENCE AND RATE OF TURNOVER OF SPHINGOMYELIN IN TISSUES OF NORMAL AND TUMOR-BEARING RATS\*

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Many studies have been made on the phospholipid concentration in rat tissues, and a number have been devoted to the rate of turnover of the phosphorus part of the phospholipid molecule. In most of these investigations no attempt has been made to distinguish between the different substances making up the total phospholipid fraction. This report will present some data on the occurrence of sphingomyelin and on the rate of exchange of the phosphorus part of its molecule. The determinations were made on liver, kidney, and spleen from normal and tumor-bearing rats.

## EXPERIMENTAL

*Animals*—Young male albino rats were used in both series. At the time of death the average weight of the seventeen normal animals was  $201 \pm 12$  (s.d.) gm. The twenty-three tumor-bearing rats referred to here are the same animals that comprised Series II of Haven and Levy (1). The average weight at death was  $239 \pm 21$  (s.d.) gm. This figure includes the weight of the tumor growing subcutaneously in the groin. Because the two series of experiments were not carried out concurrently, and because the lower weight of the normals indicates somewhat younger animals, the normal series cannot be considered the proper control for the tumor-bearing group.

*Methods*—The preparation and the administration of radioactive phosphate were carried out as described for Series II in the paper of Haven and Levy (1).

The animals were sacrificed at various times from 3 to 60 hours after the injection of the radioactive phosphate. The livers, kidneys, and spleens were removed and all fat trimmed off. In order to obtain sufficient material for determinations the kidneys from three rats were combined. A similar procedure was used for the spleens. The lipid extractions, isola-

\* Part of this work is taken from a dissertation submitted to The University of Rochester in partial fulfilment of the requirements for the degree of Doctor of Philosophy, June, 1941.

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tions, and determinations were carried out as previously described (2). Radioactivity measurements were made as described by Haven and Levy (1).

### Results

*Phospholipid and Sphingomyelin Determinations*—The quantitative values for total phospholipid and sphingomyelin are presented in Table I. In no case is there a significant difference between the values for the normal group and the corresponding figures for the animals bearing tumors. About 0.21 per cent of the wet weight of rat liver is sphingomyelin. This figure, the first for this phospholipid in rat liver, corresponds rather closely to the concentration in cat liver (2). Sphingomyelin represents a small part, approximately 6 per cent, of the phospholipid from rat liver.

TABLE I

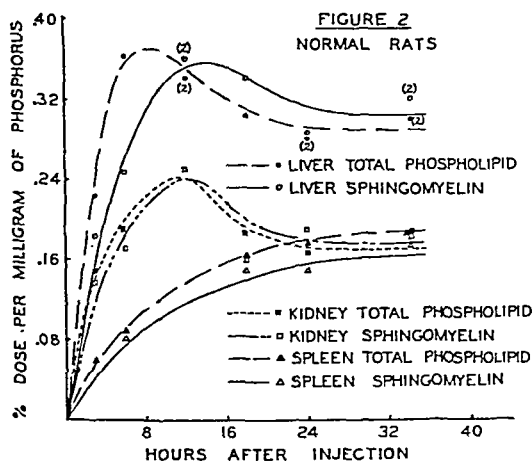
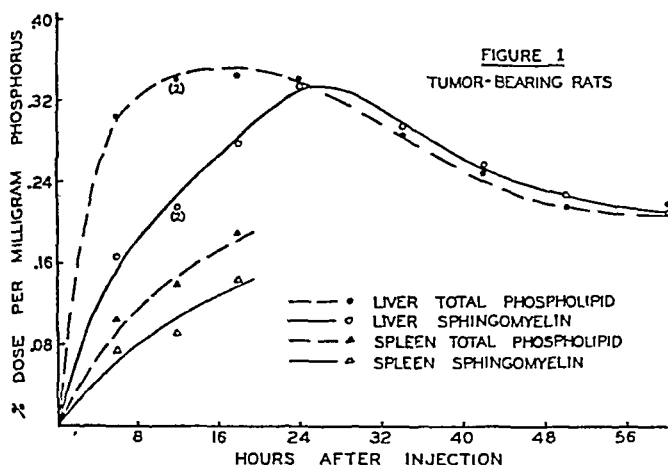
#### *Phospholipid and Sphingomyelin Concentrations in Tissues of Rats*

All values are based on the wet weight of the tissue. The averages are followed by the standard deviations.

	Tissue	No. of samples	Per cent phospholipid	No. of samples	Per cent sphingomyelin	Per cent sphingomyelin in total phospholipid
Normal rats	Liver	14	$3.45 \pm 0.39$	11	$0.20 \pm 0.032$	$5.64 \pm 0.65$
	Spleen	6	$1.51 \pm 0.17$	4	$0.35 \pm 0.030$	$24.20 \pm 1.90$
	Kidney	5	$2.77 \pm 0.07$	6	$0.63 \pm 0.050$	$22.70 \pm 2.00$
Tumor-bearing rats	Liver	23	$3.51 \pm 0.34$	23	$0.22 \pm 0.025$	$6.43 \pm 0.81$
	Spleen	3	1.73	3	0.33	18.60

The values for spleen and kidney tissue are based on a smaller number of analyses, but they indicate sphingomyelin concentrations similar to those found previously for cat tissues (2). A discussion of other values in the literature has been presented in that paper.

*Radioactivity Measurements*—Figs. 1 and 2 show the percentage of the administered dose of radioactivity which was found in the phosphorus from liver sphingomyelin after various time periods. Values for the total phospholipid mixture from liver are given for comparison. If the slope of the curves during the initial rapid rise is taken as the best indication of the rate of reaction, it may be seen that the labeled phosphate enters the sphingomyelin fraction of liver at a rate which is 50 to 65 per cent of that observed for the total phospholipid from the same tissue. The rates seem a little more rapid for the normal rats and the curves reach their peaks at an earlier hour. A somewhat similar observation has been made by Jones, Chaikoff, and Lawrence (3) using mice bearing different tumors. In the present case one should keep in mind the fact that the experiments were



FIGS. 1 AND 2. The percentage of administered radioactivity found per mg. of phospholipid or sphingomyelin phosphorus from liver, spleen, and kidney of normal and tumor-bearing rats after subcutaneous administration of a phosphate solution containing radioactive phosphorus. In the case of liver tissue each point represents an average of values from three animals unless otherwise indicated by a figure in parentheses. The values for kidney and spleen represent pooled samples.

not carried out simultaneously and that the normal rats were a little younger than the tumor-bearing animals.

As would be expected from the different rates of turnover for sphingo-

myelin and total phospholipid in the liver, the radioactive phosphate disappears more rapidly from the total phospholipid fraction when the inorganic labeled phosphate of the plasma has fallen to a low value.

The values for spleen and kidney (Figs. 1 and 2), although based on a smaller number of determinations, indicate that the rate of entrance of radioactive phosphate into the total phospholipid and sphingomyelin in these organs is definitely slower than for liver. Not too much emphasis should be placed on this observation, for the difference may be entirely due to the rate at which inorganic phosphate is able to enter the cells of these tissues as compared to liver. In the kidney the labeled phosphate seems to enter the sphingomyelin at the same rate as the total phospholipid mixture, a situation different from that seen in the liver. In the spleen the rate of turnover of sphingomyelin appears to be slightly less than that for the total phospholipid.

#### DISCUSSION

The data reported establish some values on the occurrence of sphingomyelin and indicate that the phosphate part of the molecule is replaced at a rate different from that for the other phospholipids in the case of the liver (assuming direct replacement). In the kidney the rate of turnover for sphingomyelin does not seem to be significantly different from that for the total phospholipid mixture, which consists chiefly of lecithin and cephalin. In the present study no attempt was made to separate these substances, but Chargaff and associates (4, 5) and Sinclair (6, 7) have found that in the liver the rate of formation for lecithin is usually somewhat greater than for cephalin. The same workers found equal rates of turnover for lecithin and cephalin in the kidneys. These facts suggest that the rates of synthesis of lecithin, cephalin, and sphingomyelin, while quite different in the liver, may be essentially the same in the kidney.

After most of this work had been completed, the report of Hevesy and Hahn (8) on the turnover of lecithin, cephalin, and sphingomyelin became available. The type of experiment used by these workers was quite different in some respects; yet the findings for sphingomyelin are very similar to those of the authors. Hevesy and Hahn report on the radioactivity of liver sphingomyelin from four rabbits and one hen. The animals were killed 5 and 19 hours after administration of radioactive phosphate, or after receiving continuous intravenous injection of labeled phosphate for 3, 4, and 11 hours. From the results of these experiments it is possible to calculate that the radioactivity in sphingomyelin was 30, 50, and 45 per cent of that in the total phospholipid mixture at 3, 4, and 5 hours, respectively. In the authors' experiments on rats the specific radioactivity of the liver sphingomyelin at 6 hours was about 50 per cent of that in the total phos-

pholipid in tumor-bearing animals and about 65 per cent in a group without tumors.

The rate of turnover, as measured with radioactive phosphorus, actually applies only to the phosphate part of the molecule. It is possible for the rate of turnover of fatty acids in the phospholipid molecule to be entirely different from that of the phosphate group (see Haven (9) and Sinclair (6)). In simultaneous studies with labeled fatty acids and labeled phosphate such differences would be detected. The rate of turnover for sphingomyelin, as measured with radioactive phosphorus, is fairly high. This indicates that sphingomyelin must have an active part in phospholipid phosphorus metabolism—less than the other phospholipids, but quite significant. However, it will be necessary to determine the rate of exchange of fatty acids in the molecule before definite conclusions concerning its rôle in fatty acid metabolism can be made.

#### SUMMARY

The sphingomyelin concentrations in the liver, kidney, and spleen from normal and tumor-bearing rats were determined. The values, expressed as per cent of the wet weight of tissue, are as follows: liver from normal animals 0.20, liver from tumor-bearing animals 0.22, kidney from normals 0.63, spleen from normals 0.35, spleen from tumor-bearing animals 0.33.

Measurements on the entrance of radioactive phosphate into sphingomyelin indicate that the rate of synthesis for this phospholipid is slower than for the other phospholipids in the liver, but approximately equal to the others in the kidney.

The authors are indebted to members of the Radiology and the Physics Departments of The University of Rochester for the preparation of the radioactive phosphorus.

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# ELECTROPHORETIC COMPONENTS OF GLOBIN

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As a rule, globin is prepared from crystalline or purified hemoglobin solutions by treatment with acid or acid acetone. Anson and Mirsky (1) have shown that by this method globin hydrochloride is obtained from which a native globin solution can be prepared by careful neutralization. Roche (2) and Johnson and Bradley (3) have further purified native globin by salting-out with ammonium sulfate. Roche and Combette (4) found that native globin solutions contain one component which can be precipitated by 55 per cent saturation with ammonium sulfate and another component which is precipitated at between 55 and 65 per cent saturation.

According to Svedberg (5), globin is a polydisperse protein and does not have a definite molecular weight. A minimum molecular weight of 17,000 was assigned to globin, suggesting that hemoglobin is composed of four globin units. Polson (6) found that the molecular weight of myoglobin is 17,600. There is evidence in the literature suggesting both the existence (7, 8) and absence (9, 10) of more than one hemoglobin in the same animal. Landsteiner, Longsworth, and van der Scheer (8) have recently identified two electrophoretic components in crystalline chicken hemoglobin. In this paper we wish to report on the electrophoretic analysis of globin solutions.

*Electrophoresis Experiments*—Native globin was prepared from beef blood as described by Anson and Mirsky (1). A solution was obtained which contained 1.8 per cent protein as calculated from nitrogen determinations. (The nitrogen content of globin was found to be  $16.8 \pm 0.1$  per cent from an average of four determinations. Roche, Roche, Adair, and Adair (11) calculated the nitrogen content of beef globin from nitrogen determination of hemoglobin to be 17.06 per cent.) The solution was diluted with a 0.10 M glycine-hydrochloric acid buffer containing about 0.12 M sodium chloride to yield a 0.5 per cent globin solution, and then dialyzed against the buffer medium until the hydrogen ion concentrations and conductivities were nearly identical. This native globin solution was tested by the Tiselius method at  $1.0^\circ$  at pH 2.6 and at pH 3.7 and showed in both instances the presence of two components: a "slow" component consisting of approximately 40 per cent of the material and having an

average mobility of  $6.6 \times 10^{-5}$  at pH 2.6 and  $5.0 \times 10^{-5}$  at pH 3.7 and a "fast" component consisting of approximately 60 per cent of the material and having a mobility of  $9.4 \times 10^{-5}$  at pH 2.6 and  $7.1 \times 10^{-5}$  at pH 3.7. To investigate the possibility that one of these components is denatured globin which remained in solution when the native globin was prepared, an unpurified globin preparation containing a large percentage of denatured globin was subjected to electrophoretic analysis. Again the same components were found and in addition a small amount of a third very slow component. The composition was 32 per cent of the slow and 68 per cent of the fast component, which is very similar to the composition of native globin. We next investigated the presence of these two components in a globin preparation which was prepared from native globin by reprecipitating it with acid acetone and then washing the precipitate with acetone and ether. This preparation contained very little water-soluble (na-

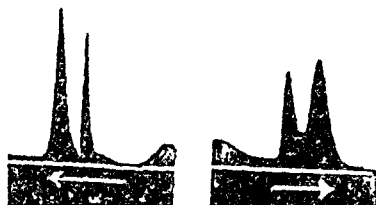


FIG. 1. Longworth patterns of native globin fraction precipitated at between 55 and 65 per cent saturation with ammonium sulfate.

tive) globin; however, it again contained 40 per cent of the slow fraction migrating with a mobility of  $6.4 \times 10^{-5}$  at pH 2.5 and 60 per cent of a fast component migrating with a mobility of  $9.1 \times 10^{-5}$  at the same pH.

Since we wished to investigate these components analytically and also their physical properties, we attempted to separate the fractions by salting-out methods. A fraction described by Roche and Combette (4) precipitating at between 55 and 65 per cent saturation with ammonium sulfate was prepared. After 2 weeks of continuous dialysis first against distilled water and then against a buffer solution, this fraction was tested (*cf.* Fig. 1) and was found to contain the same two components in the same proportion.

*Dissociation of Hydrogen Ions*—The fractions were then separated by electrophoresis in the large cell apparatus. They were dialyzed until practically chloride-free and their acid-binding capacity was determined from pH 5.5 to 6.8. The average slope was  $1.25 \pm 0.12$  for the slow fraction and  $0.96 \pm 0.05$  for the fast fraction in equivalents per mg. of nitrogen  $\times 10^6$ . It is of interest to note that the fraction which has a

higher mobility at the range of pH 2.5 to 3.7 possesses less buffering capacity in the pH range of 5.5 to 6.8.

*Absorption Spectra*—Roche, Dubouloz, and Jean (12) studied the ultra-violet absorption spectra of globins prepared from various mammalian hemoglobins and found not only species differences but also individual differences. We determined the absorption spectra of fast and slow globin components in solutions containing 0.0857 per cent globin in glycine-hydrochloric acid buffer having a pH of 2.5, and found that the maximum extinction of the bands occurs at the same wave-lengths, but that the extinction for the fast component is somewhat greater than that for the slow component.

*Sulfur Determinations*—In these we were especially handicapped by the fact that only small quantities of the components were available. The determinations were carried out by burning the dried protein in a slow stream of oxygen and collecting the gases on a glass spiral moistened with hydrogen peroxide. The spiral and the boat were washed with distilled water and the washings concentrated after being acidified with trichloroacetic acid. The sulfate in the solution was determined in a manner similar to that used by Marenzi and Banfi (13). When large quantities were available, as in the case of whole or native globin, Fiske's benzidine acidimetric method (14) was used. From the average of ten determinations, the sulfur content of native and whole globin was found to be  $0.61 \pm 0.02$  per cent of free globin. This is in good agreement with the values given by Kaiser (15). Sulfur to nitrogen ratios were determined for the components. The nitrogen of the fast component was not significantly different from that of the whole globin; therefore, the nitrogen content of the slow component must be the same, that is, 16.8 per cent. From eight determinations, the fast component was found to contain  $0.78 \pm 0.03$  per cent sulfur. On the basis of the relative proportions of fast and slow components as determined by electrophoretic analysis, the sulfur content of the slow component is calculated to be  $0.36 \pm 0.04$  per cent. Actual determination on four small samples, each less than 10 mg., gave an average value of 0.49 per cent. This value, however, is rather uncertain.

#### DISCUSSION

Although there is evidence for the existence of different hemoglobin molecules in crystals prepared from the blood of the same animal (7, 8), two hemoglobins were not isolated from their solutions. The globin components were both found in such substantial proportions that they could hardly be considered as deriving from two different hemoglobins present in these proportions in cattle blood. Evidence for the existence of two hemoglobins in these proportions has not been encountered in spite

of rather extensive investigation of cattle hemoglobin. It is not probable that the components are incidental denaturation products formed at the cleavage of hemoglobin. The conditions under which the various preparations were obtained were very different; yet the proportions of the two components were found to be the same. This, together with the fact that the chemical composition (sulfur to nitrogen ratio) of the two fractions is different, suggests that the globin components isolated are either derived from two different components of a complex protein or are well defined distinct split-products of a protein.

The bearing of these findings on the structure of hemoglobin is being investigated further. However, the hypothesis that each of the four iron-porphyrin groups present in hemoglobin is attached to 1 globin molecule (16) having the molecular weight of about 16,500 or to distinct components of a complex globin molecule has merits. If one assumes that the globin components isolated experimentally were derived from small globin units, four of which exist in each hemoglobin molecule, then one will have to conclude that the two components are not equally distributed in each hemoglobin molecule since, if they were, their ratio should be either 1:3 or 2:2 and not the one found, 2:3. This ratio can, however, be explained by assuming that the globin components are distributed statistically in the hemoglobin molecules and that molecules having the composition of *aabb*, *aaab*, *abbb*, and even *aaaa* and *bbbb* exist, where *a* and *b* are used to designate the two globin components. Since the occurrence of the one component, *a*, is 60 per cent and that of the other, *b*, is 40 per cent, the majority of the hemoglobin molecules should contain the complex globin *aabb*, a substantial number should have the constitution *aaab*, and somewhat fewer should have the constitution *abbb*; the other forms mentioned should occur very rarely. Obviously the sulfur content of these various molecules should be different, since if the average sulfur content of the hemoglobin molecule is 12.5 atoms, the molecular weight of heme-free hemoglobin being taken as 65,600, the fast component should contain 4 atoms of sulfur, that is 0.78 per cent, and the slow component, 2 atoms of sulfur, that is 0.39 per cent. Work is being continued on this aspect of the problem.

#### SUMMARY

In various globin fractions, which behave rather differently with regard to their solubility, the same two electrophoretic components were demonstrated. There is about 60 per cent of the fast component and 40 per cent of the slow component in globin.

The components were isolated and studied. They were found to be different with respect to their hydrogen dissociation curve, their absorption spectra, and their sulfur content.

The authors wish to express their appreciation to Miss Helen Sikorski for her assistance in the electrophoretic analysis.

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# DETERMINATION OF THIAMINE IN URINE BY THE THIOCHROME METHOD: ESTIMATION OF THE BLANK

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The thiochrome method of Hennessy and Cerecedo (1, 2) for the determination of thiamine has been used for several years in the laboratories of the Division of Biochemistry at the Mayo Clinic for the determination of thiamine in urine. We have demonstrated to our satisfaction that thiamine added to urine can be quantitatively recovered by this method. However, it has not been established with certainty that the initial value represents the true thiamine content of the urine as excreted. We have pointed out previously (3) that when large amounts of nicotinic acid are ingested, the urine contains non-thiamine material which acts very much like thiamine in the method and causes the apparent value for thiamine to be too high. Ordinarily, the amount of this non-thiamine material is too small to interfere seriously with the determination, but, since it is sometimes desirable to know the quantity of thiamine that is excreted when large amounts of nicotinic acid are being administered, and since in many other conditions it would be desirable to determine how much of the apparent thiamine is attributable to other substances, this study was undertaken.

A partial value for the non-thiamine material was previously obtained by measurement of the fluorescence before and after destruction of the thiochrome with ultraviolet light. There was some reason, however, for belief that this procedure also destroyed some of the non-thiamine fluorescence, and it was not very satisfactory. It seemed probable that the thiamine could be destroyed by sulfite (4) and that the non-thiamine material which acted like thiamine in the method could then be determined by the usual procedure for thiamine. The results herein presented support the conclusion that this procedure affords a valid correction of the values for thiamine in the urine determined by the thiochrome method.

Since the procedure of Hennessy and Cerecedo has been modified somewhat to adapt it to large scale use, a brief outline of the procedure will be given so that certain later steps may be made clear.

The reagents and tubes for permutit are those described by Hennessy

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(2) and the permutit is purified according to his description. It is believed advisable to charge the tubes with 5 cm. of fresh permutit for each determination, although it is possible to use the permutit a number of times. Urine is collected in a bottle which contains 10 cc. of glacial acetic acid. The aliquot for analysis, which is chosen to contain approximately 1  $\gamma$  of thiamine, is adjusted to a pH of between 4 and 4.5. The amount of glacial acetic acid or of saturated solution of sodium acetate necessary for adjustment of the pH is determined with a separate aliquot, and brom-cresol green is used as the indicator. Even though the thiamine content of the urine is very low, the volume of the aliquot is restricted to 20 ml. to avoid the accumulation of salts by the column. After passage of the urine, the column is washed with 30 ml. of distilled water and the thiamine is then removed by passage through the column of a hot 25 per cent solution of potassium chloride in approximately 0.1 N hydrochloric acid until 15 ml. have collected in a graduate. The entire solution containing the thiamine is transferred to a test-tube 2.5 by 20 cm. in size and the graduate is rinsed once with 2 ml. of water. After the addition of 17 ml. of isobutanol, the mixture is stirred with a stream of air for a few seconds until it has the appearance of a homogeneous emulsion. Then 2 ml. of 10 N sodium hydroxide containing 6.6 mg. of potassium ferrieyanide are added quickly from a pipette which is filled and emptied by means of a rubber bulb. Stirring is continued for 1 minute. The aeration tube is washed down with distilled water and the tube is set aside to allow the two phases to separate. Only occasionally does a stable emulsion form, but in case this happens, the emulsion is broken by centrifugation. The addition of the alkali and subsequent stirring are critical steps in the process, and a rigid technique must be developed to achieve constant duplication of results. The isobutanol layer is transferred to another similar test-tube by means of suction. A little anhydrous sodium sulfate is added and shaken up with the isobutanol to remove turbidity. When the salt has settled, 10 ml. of isobutanol are withdrawn for fluorometry.

This procedure is well adapted for large scale routine determinations. It also provides for complete duplicates instead of duplication of the oxidative procedure on aliquots of the potassium chloride solution obtained by passage of a single specimen of urine through a column of permutit. Provision for a blank determination at the point at which alkali is added by elimination of the ferrieyanide is of no value when it is applied to urine, as we have shown.

In the present study of the blank determination the conditions necessary for complete destruction of thiamine were established. The data in Table I show that at pH 5, 10  $\gamma$  of thiamine in 10 to 15 ml. of urine were completely destroyed by heating for 15 minutes at a temperature of 100° with 25 mg. of sodium sulfite. Destruction of the 10  $\gamma$  of added thiamine was

not quite complete in the volume of 20 ml.; approximately 1 per cent (0.13  $\gamma$ ) of the added thiamine remained after the treatment with sulfite (Table I, lines 9 and 10). However, since 10  $\gamma$  represent 5 to 10 times as much thiamine as would ordinarily be encountered in this volume of urine, 25 mg. of sulfite afford an adequate margin of safety. The data of Table

TABLE I  
*Results of Variations in Amount of Sulfite and in Duration of Heating*

Volume of urine	Thiamine added	Weight of sulfite	Time heated at 100°	Blank value in volume used in terms of thiamine
ml.	$\gamma$	mg.	min.	$\gamma$
10		25	15	0.23
10		25	30	0.21
10		50	15	0.25
10		50	30	0.24
10	10	25	15	0.45
10		25	15	0.46
15		25	15	0.48
15	10	25	15	0.48
20	10	25	15	0.53
20		25	15	0.66

TABLE II  
*Variation of Blank Value with Volume*

Urine, 24 hr. specimen No.	Volume of sample	Blank value in terms of thiamine
	ml.	$\gamma$
1	10	7.8
1	20	4.9
2	10	10.9
2	20	6.0
3	10	5.3
3	20	3.8
4	5	11.0
4	10	7.8
4	20	5.2

I were obtained with two different specimens of urine from persons who were ingesting 300 mg. of nicotinic acid daily.

It will be observed that the blank values do not increase in proportion to the volume of urine; that is, the value found in 15 ml. of urine is not significantly greater than the value found in 10 ml. of urine. This effect is shown more specifically in Table II, in which the blank values are given as the total amounts in 24 hour specimens. Since the blank value is not

proportional to the volume of urine, the aliquot used for the blank determination must be the same as that used for the determination of the total apparent thiamine.

The following procedure has been found satisfactory for the determination of the non-thiamine material which occurs in urine and which is determined along with thiamine in the usual procedure. The desired volume of urine, 5 to 20 ml., is adjusted to approximately pH 5 by the addition of a few drops of a saturated solution of either sodium acetate or of glacial acetic acid. Then 25 mg. of sodium sulfite are added, either as the anhydrous salt or in 1 ml. of a solution, and the mixture is heated in boiling water for 15 minutes. The pH of the urine may change somewhat during

TABLE III

*Comparison of Blanks Obtained by Destruction of Thiamine with Sulfite and Those Obtained by Destruction of Thiochrome with Ultraviolet Light*

Urine, 24 hr. specimen No.	Residual values, micrograms thiamine after		
	Use of sulfite	Destruction of thiochrome	Irradiation after sulfite
1	8.8	7.2	
2	17.6	16.8	
3	8.0	8.0	
4	5.2	5.3	
5	5.1	5.1	
6	6.5	5.1	5.1
7	4.3	4.1	3.5
8	7.1	5.3	5.5
9	9.8	9.0	8.8
10	5.0	5.0	4.7

the heating, but does not do so sufficiently to interfere with either the destruction of thiamine or the recovery of material by the permutit. Without further treatment the urine, either hot or cold, is passed through the column of permutit and thereafter the procedure is carried out as usual.

Comparison in Table III of the blank values obtained with the use of sulfite (second column) and those obtained by destruction of the thiochrome with ultraviolet light (third column) reveals agreement, on the whole, with higher values for the sulfite blank in some instances. Although it may appear that not all the thiamine was destroyed in the latter instances, such an explanation seems unlikely, since the addition of more sulfite and maintenance of a longer period of heating resulted in the same values. Irradiation with ultraviolet light of the isobutanol solution after the use of sulfite usually lowered the value of the sulfite blank to the same value as was obtained by irradiation of the thiochrome solution. Since

the irradiations necessarily took some time and could not all be carried out in 1 day, there were small fluctuations in the fluorometer readings because of changes in the intensity of the ultraviolet light. In view of this difficulty agreement is considered satisfactory for urine Specimens 6 to 10 (Table III). The agreement between the blank values obtained by the two methods supports the conclusion that sulfite destroys only thiamine, so far as the method is concerned, and that it does not act on any other constituent of the urine to change the amount of fluorescence exhibited by the non-thiamine material which acts like thiamine in the method.

TABLE IV

*Typical Results of Analysis of Urine for Thiamine, Including Blank Values*

Patient No.	Total volume of urine	Aliquot	Total apparent thiamine	Blank in terms of thiamine	Net thiamine
	<i>ml.</i>	<i>ml.</i>	$\gamma$	$\gamma$	$\gamma$
1 a	3240	20	117.0	19.0	98.0
1 b	2840	20	237.0	15.0	222.0
2	670	5	87.0	13.0	74.0
3 a	1600	10	186.0	8.0	178.0
3 b	1680	15	130.0	9.0	121.0
4*	500	20	7.0	1.8	5.2
5	600	20	5.7	2.9	2.8
6 a	900	20	10.0	5.2	4.8
6 b	740	20	11.0	3.7	7.3
7	940	20	8.6	4.5	4.1

\* Patients 4 to 7 were on a diet restricted in thiamine content.

In Table IV are presented some typical results for total apparent thiamine, for the blank values, and for net thiamine obtained with various specimens of urine. Unless nicotinic acid therapy is under way, the blank values are small as compared to the total value, unless the total value itself is very small. In that case the blank value may be 50 per cent or more of the total value. It appears that, even when the intake of thiamine is severely restricted, a few micrograms of thiamine are excreted each day.

## SUMMARY

Conditions have been established for the complete destruction of thiamine in quantities of urine ordinarily used for the determination of thiamine. The residual fluorescence of the urine as determined by the usual thiochrome procedure gives a blank value which affords a valid correction of the gross value for thiamine.

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# A DIRECT COLORIMETRIC METHOD FOR THE DETERMINATION OF UREA IN BLOOD AND URINE

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At the present time urea is usually determined by one of three methods; viz., the aeration and titration procedure of Van Slyke and Cullen (1), the gasometric technique of Van Slyke (2), or one of the many modifications of the nesslerization procedure. The Van Slyke and Cullen procedure is accurate but requires 3 ml. of whole blood and is time-consuming; the gasometric method is also accurate but requires special equipment which is often not available in clinical laboratories. The chief fault of the nesslerization procedures is their inaccuracy, due primarily to the readiness with which the final solution becomes turbid and to a difference in quality of the color produced in the standard and sample. Gentzkow (3) claims to have overcome these errors in a recent modification of the direct nesslerization technique. All of these methods, with the exception of the gasometric procedure, also suffer from the disadvantage that ammonia is an interfering substance and must be determined separately.

The method to be described has the following advantages: (1) it is a colorimetric reaction which is applied directly to urine or to blood filtrate, no aeration or distillation being necessary; (2) ammonia does not interfere with the reaction; (3) a degree of accuracy is possible which surpasses that of most nesslerization procedures and is at least equal to that of the Van Slyke techniques; (4) the method is believed to be the simplest and most rapid yet described.

The method is based on a reaction first described by Fearon (4). When urea is heated with biacetyl monoxime in acid solution, a yellow color develops, deepening on subsequent oxidation with potassium persulfate. Many substituted ureas give a red color, but only urea yields a yellow pigment. Fearon used the reaction for the approximate determination of citrulline in casein, Gornall and Hunter (5) later modifying the procedure for the accurate determination of citrulline in tissues. Abelin (6) applied the reaction to the determination of urea in serum, but, since the reaction was carried out in neutral solution and no oxidizing agent was added, only a very rough approximation to the amount of urea present was given.

Fearon (4) has studied the specificity of the reaction. Urea was the

only substance giving a yellow to orange color but the following compounds gave rise to a red color, similar to that obtained with citrulline: methylurea, butylurea, phenylurea,  $\beta$ -naphthylurea, dimethylurea, allantoin, semicarbazide, citrulline, and all higher proteins examined. The test was found by Fearon to be negative with ammonium salts, hydrazine, carbamate, cyanate, acetamide, diphenylurea, guanidine, methylguanidine, creatinine, creatine, glycoeyamine, uroxamic acid, uric acid, indole, and all amino acids examined (glycine, glycine ester, sarcosine, alanine, cystine, tyrosine, tryptophane, arginine, histidine, lysine, proline, hydroxyproline, asparagine, aspartic acid). Fearon (4) concluded that "the test is positive with compounds containing the system  $R_1 \cdot NH \cdot CO \cdot NH \cdot R_2$ , where  $R_1$  is either hydrogen or a simple aliphatic radicle, and  $R_2$  is not an acyl radicle." Gornall and Hunter (5) added some  $\alpha$ -carbamido derivatives to the list of substances giving a positive test. Allantoin has been further tested in our own laboratory and was found to give only a very feeble positive reaction. Although human urine was used throughout in the experiments to be described, the feeble color given by allantoin in animal urines would introduce no appreciable error. The small amount of color introduced by allantoin would be further minimized by the use of the proper filter in the colorimeter.

#### EXPERIMENTAL

The method given below was found to be that which would combine an optimum stability with an optimum intensity of color. The conditions necessary for maximum color development from urea are very similar to, although not identical with, those which are used in the determination of citrulline (5).

#### *Method*

##### *Reagents—*

1. Concentrated HCl.
2. Biacetyl monoxime; a 3 per cent aqueous solution. This solution appears to last indefinitely when kept in the refrigerator.
3. Potassium persulfate; a 1 per cent aqueous solution. The solution is somewhat unstable, and should be made up weekly and kept in the refrigerator. Actually it is good for a somewhat longer period, but solutions 4 weeks old were found to give definitely lower results than did those which were freshly prepared.

4. Standard urea solution. A solution was prepared such that 1 ml. contained 0.1 mg. of urea. A little chloroform was added as a preservative.

*Procedure—*The volume of sample used should contain preferably from 0.1 to 0.2 mg. of urea (extreme limits, 0.05 to 0.3 mg.) and must not exceed 3 ml. For most samples of human urine 0.01 ml. (1 ml. of a 1:100

dilution), and for blood 3 ml. of the usual Folin-Wu filtrate were sufficient. Place appropriate volumes of sample in test-tubes, 1 ml. and 2 ml. of urea standard in similar tubes, and 3 ml. of distilled water in another tube to serve as a blank. The volume in each tube is then made up to 3 ml. with distilled water.

Add to each tube 5 ml. (accurately measured) of concentrated HCl. Follow this with 0.5 ml. of 3 per cent biacetyl monoxime.

Mix the contents by rotation and place the tubes in a vigorously boiling water bath for *exactly* 10 minutes. During the heating it is necessary to prevent evaporation. This may be accomplished by covering the tubes with hollow glass bulbs, small funnels, or some similar device. In this laboratory ordinary glass marbles were used successfully.

Remove the tubes simultaneously and cool for 2 minutes in running water.

Add slowly 0.25 ml. of 1 per cent potassium persulfate, so that a separate layer is formed. After this reagent has been added to all of the tubes, stopper, and mix simultaneously by inverting a few times.

The intensity of color is then read at intervals in a Klett photoelectric colorimeter, with a No. 42 filter. The time required for development of the maximum color depends on the concentration of urea. If the sample contains 0.1 mg. of urea, the maximum develops in about 5 minutes after mixing with the persulfate; 0.2 mg. requires 10 to 15 minutes, and 0.3 mg. from 25 to 30 minutes. Since the color fades at a very slow rate, it is sufficient, for most purposes, to take readings at, say, 5, 15, and 25 minutes. With a little experience one can easily judge the approximate time at which the maximum will occur by noting the color of the tubes when removed from the water bath.

The results may be calculated in the usual way by reference to the reading of the standard. Since, however, the color-concentration curve is not a straight line throughout the whole range, some error is introduced by this method of calculation if the standard and unknown readings are too far apart. Better results are obtained if the values are taken from a calibration curve (Fig. 1). Although the weekly changes in persulfate solution have no effect on the curve, it was found that a new curve had to be constructed for each solution of biacetyl monoxime. Fortunately the latter reagent is stable and can be prepared in large amounts, so that one curve will last for a long period.

The method as outlined was designed for use with the Klett photoelectric colorimeter, but results sufficiently accurate for clinical use can be obtained with the ordinary visual colorimeters.

*Comparison with the Van Slyke-Cullen Method.* *Urine Analyses*—Table I shows the results on a number of urines analyzed by both methods. The values are in good agreement, especially when results by the biacetyl



monoxime method are obtained from the calibration curve. In subsequent tables all values given were obtained by reference to the curve.

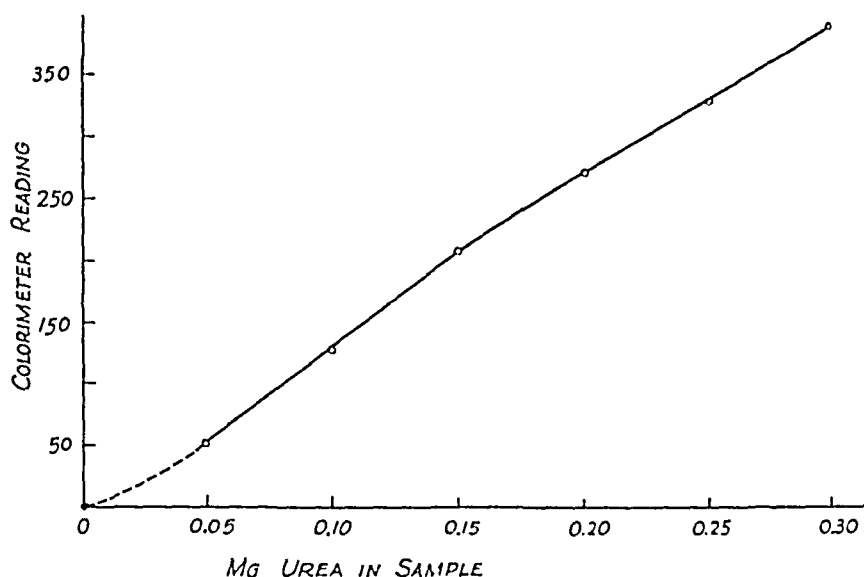


FIG. 1. Calibration curve showing the relation between the amount of urea in the sample and readings on the Klett photoelectric colorimeter, with No. 42 filter.

TABLE I

*Comparison of Biacetyl Monoxime Method with Van Slyke-Cullen Procedure for Determination of Urea in Urine*

All values are expressed as gm. of urea per 100 ml. of urine.

Specimen No.	Van Slyke-Cullen method	Biacetyl monoxime method	
		Calculated from standard	From curve
1	2.50	2.51	2.54
2	2.16	2.12	2.13
3	2.26	2.21	2.26
4	2.82	2.90	2.82
5	2.51	2.65	2.58
6	2.21	2.26	2.18

*Blood Analyses*—The values found by the two methods did not agree as closely for blood as had been the case with urine (Table II). It was felt, however, that, at least in our hands, the more accurate results were those obtained with the biacetyl monoxime method. In the first place, checks on duplicate samples showed much better agreement by the present

method than by the Van Slyke-Cullen procedure. Secondly, excellent recoveries of added urea were obtained by the biacetyl monoxime method and, finally, the potential errors involved in the application of the Van Slyke-Cullen technique to blood analysis are certainly greater than when that method is used for urine, even though Van Slyke (2) was able to obtain the same results from whole blood and from the Folin-Wu filtrate, using the aeration and titration method in the one case and the gasometric technique in the other. Cells of whole blood have been shown to contain arginase (7, 8). With sufficient time, there will be some urea formation from the action of this arginase on the arginine of the blood. During the incubation of whole blood with urease one must, therefore, strike a happy medium between too short an incubation, giving low results because of incomplete conversion of urea to ammonia and  $\text{CO}_2$ , and too

TABLE II

*Comparison of Biacetyl Monoxime Method with Van Slyke-Cullen Procedure for Determination of Urea in Blood*

All values are expressed as mg. of urea per 100 ml. of whole blood.

Specimen No.	Van Slyke-Cullen method	Biacetyl monoxime method
1	35.2	34.2
2	29.5	28.0
3	29.8	30.0
4	34.7	36.5
5	34.1	34.2
6	21.6	21.2
7	40.1	40.4

long an incubation, giving high results because of the formation of urea from arginine.

Therefore, whereas at least 15 minutes (we have used 30) are advised for the incubation of urease with urine, only 5 minutes are recommended for blood. In a study of the effect of blood cells on the determination of urea by the Van Slyke-Cullen method, Behre (8) concluded that the true urea content of blood can be obtained only by the analysis of blood filtrates. It must be admitted that the color obtained with blood filtrates treated with biacetyl monoxime is slightly different, qualitatively, than that obtained with the standard urea solution, owing to the presence of small amounts of citrulline or other material giving the carbamido reaction. The colors given by urea and by carbamido compounds are quite different. Fig. 2 shows the per cent transmission of light between 400 and 650  $\text{m}\mu$ , as determined with the Coleman spectrophotometer, for the colors produced by urea and by citrulline. The No. 42 filter, supplied with the Klett

photoelectric colorimeter, allows passage of light between 400 and 465  $m\mu$ . It is in this range that the two absorption curves diverge most. The use of this filter, then, eliminates a considerable part of the small error introduced by the foreign color. If this color were the source of the lack of agreement between the two methods, the results by the present method should be consistently higher than those obtained by the Van Slyke-Cullen procedure, and this was not the case.

Some preliminary work with the Coleman spectrophotometer indicated that this instrument offers definite advantages when the present method

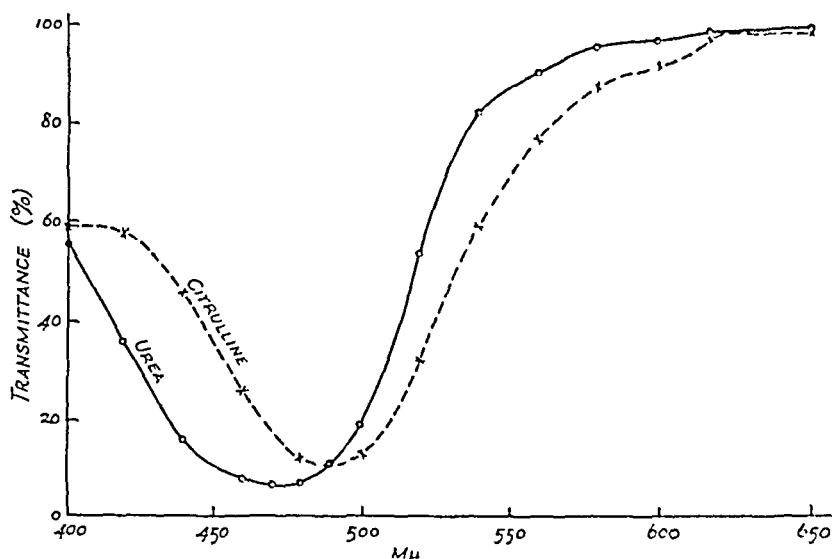


FIG. 2. Curves showing the per cent transmission of light between 400 and 650  $m\mu$  by colored solutions obtained by treating urea and citrulline with biacetyl monoxime in acid solution, followed by persulfate.

is applied to blood analysis. The method becomes more sensitive; so that low blood ureas, which are near the lower limit for the method as outlined above, give satisfactory readings, and the foreign color found in blood filtrates is perhaps more adequately eliminated by the use of a more nearly monochromatic light source.

*Recovery of Added Urea*—Recovery of urea added to urine is shown in Table III. In the last three specimens the urine was treated with urease to remove urea already present, the urease then being removed by acidifying, boiling, and filtering the urine. It was found, however, both by the present method and by the Van Slyke-Cullen procedure that the resulting urine still contained 28 mg. of urea per 100 ml.

Recovery of urea added to blood is shown in Table IV. The original urea content of the blood is much lower than in the samples of Table II. The blood specimens of Table II were mixed samples, some being pathological, from the clinical chemistry laboratory, whereas those of Table IV were from normal individuals.

TABLE III  
*Recovery of Urea Added to Urine*

All results are expressed as mg. of urea per 100 ml. of urine.

Specimen No.	Urea originally present	Urea added	Urea found	Per cent of theoretical
1	1710	211	1940	101.6
1	1710	306	2065	102.4
2	1360	518	1864	99.2
3	2258	289	2516	98.8
4	1750	521	2214	97.5
5	28*	1626	1656	100.1
5	28*	1832	1884	101.3
5	28*	2027	2088	101.6

\* Urea largely removed by treatment with urease.

TABLE IV  
*Recovery of Urea Added to Blood*

All results are expressed as mg. of urea per 100 ml. of whole blood.

Specimen No.	Urea originally present	Urea added	Urea found	Per cent of theoretical
1	20.5	7.5	28.1	100.4
2	20.3	15.0	36.1	102.3
2	20.3	25.0	45.2	99.8
3	27.8	15.0	42.6	99.5
4	26.8	20.0	47.8	102.2
5	20.8	20.0	40.6	99.5

*Time of Maximum Color Development*—The rate of development of color with various concentrations of urea is indicated in Fig. 3. The time required to reach the maximum color is seen to increase with the concentration of urea. If it is necessary to obtain the greatest possible accuracy, successive readings must be taken frequently enough so that the true maximum will be observed. Since, however, the rate of fading is slow, it is possible to obtain fairly accurate results, suitable for most purposes, by taking readings at 5 and 10 minutes when the concentration appears low, and at 20 and 30 minutes for samples with a higher concentration of urea.

*Effect of Time of Heating*—The amount of color developed is dependent on the length of time in which the tubes are in the boiling water bath. The intensity of color increases rapidly up to 9 minutes boiling, and then increases more slowly. 10 minutes were selected as a reasonable length of time which would give nearly the maximum amount of color obtainable. In order to get reproducible results it is necessary to adhere strictly to a given time of heating. In addition to this it is also advisable to reproduce as closely as possible the other conditions of heating. To this end we have used test-tubes of a uniform diameter and have avoided superheating by employing a wire rack which supports the tubes vertically, about 2 inches

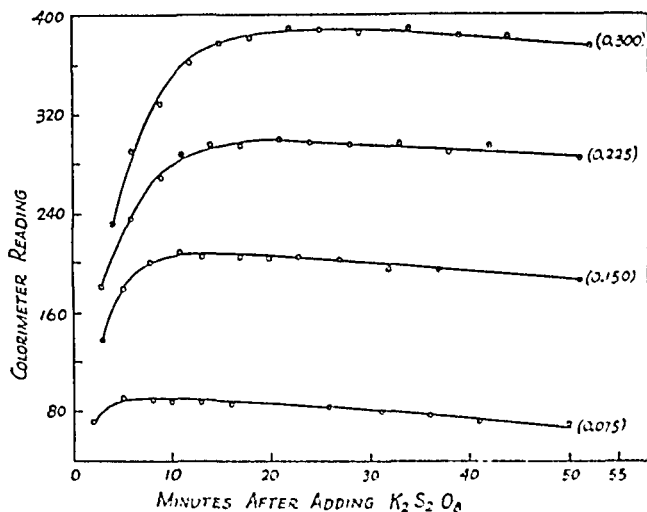


FIG. 3. Curves showing the relationship between colorimeter readings and time elapsed after addition of persulfate for various concentrations of urea. The figures in parentheses indicate the number of mg. of urea in the sample.

from the bottom of a large water bath. The water in the bath must, of course, be boiling vigorously throughout the entire heating period. Lower temperatures ( $37^\circ$ ,  $45^\circ$ ,  $60^\circ$ ) were found to give such a slow development of color as to be entirely impracticable.

*Amount and Kind of Oxidizing Agent*—Oxidation is necessary in order to get the maximum intensity of color, but an excess of oxidizing agent will cause a rapid destruction of the pigment. Fearon found that, whereas most of the commoner oxidizing agents would develop the color, potassium persulfate was less destructive to the formed pigment. Different amounts of persulfate were tried and it was determined that the optimum effect, maximum color with slowest fading, was obtained by using 0.25 ml. of a 1 per cent solution.

*Effect of Light*—During the early part of this work a set of tubes was inadvertently placed, after addition of persulfate, in the direct sunlight, and was found to fade very rapidly. Further experiments disclosed that only direct sunlight had this effect. Curves, showing development of color against time, constructed for samples carried through the procedure on a bright day in the ordinary light of the laboratory were identical with curves for other samples carried through the procedure in darkness, the latter being brought into the light only for the addition of reagents. When the samples are placed in the sunlight *after* the addition of persulfate, the only effect noted is a rapid fading. If, however, two similar sets of tubes are carried through the procedure, one set being placed in a boiling water bath in direct sunlight, while the other is heated in the ordinary light of the laboratory, it is readily seen that the effect of sunlight at this stage is to *increase* the color, the deep yellow color produced being apparently the same as that formed on the addition of persulfate in the regular procedure. This color, however, fades very rapidly and it was not possible to determine the maximum intensity by colorimetric readings. It appears, then, as if direct sunlight has, at least qualitatively, the same effect as does the addition of an excess of potassium persulfate.

*Other Factors*—The amount of biacetyl monoxime solution to be used was determined experimentally and 0.5 ml. of a 3 per cent solution was found to give the best results. Most samples of biacetyl monoxime are not pure white but are slightly tinged with yellow. The amount of yellow color may increase in stored bottles. Apparently this has no effect on the determinations, the foreign color being taken care of by the blank. It was found, however, that the calibration curve changed materially when a new batch of biacetyl monoxime solution was prepared. However, as mentioned previously, the reagent is stable and can be prepared in rather large amounts, thus obviating the necessity of constructing new calibration curves at frequent intervals.

Fearon (4) states that " $\text{H}_2\text{SO}_4$ ,  $\text{H}_3\text{PO}_4$  or trichloroacetic acid may be used as condensing agents instead of  $\text{HCl}$ , but appear to offer no advantages." This was with reference to the color produced with citrulline. We have found that the only acid which could be substituted for  $\text{HCl}$  in the determination of urea was sulfuric acid, and that even in this case the intensity of color was distinctly less than when  $\text{HCl}$  was used. The quantities of sample and acid employed in the procedure given above were designed as being particularly suited for the Klett photoelectric colorimeter.

#### SUMMARY

A direct colorimetric procedure for the determination of urea in blood filtrates and in urine has been presented.

The procedure is based on the reaction between urea and biacetyl monoxime in acid solution to give a yellow color, deepening to a yellow-orange on subsequent oxidation with potassium persulfate.

The method is believed to possess the following advantages: (1) it is rapidly and simply carried out, requiring no special apparatus or particular degree of skill; (2) the determination can be carried out with 3 ml. of blood filtrate; (3) ammonia offers no interference; and (4) the degree of accuracy compares well with that of the best previously published procedures.

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## SPECTROPHOTOMETRIC STUDIES

### X. STRUCTURAL INTERPRETATION OF THE SPECTRA OF CYANIDE, PYRIDINE, AND CARBON MONOXIDE DERIVATIVES OF CYTOCHROME C AND HEMOGLOBIN

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The investigations of Theorell (1) have led to the conclusion that in cytochrome *c* 2 histidine and 2 cysteine residues of the protein are concerned in its union with the prosthetic group. Both imidazole groups coordinate strongly with the iron, while the cysteine molecules combine in some type of thio-ether linkage with the vinyl residues of the cytochrome *c* hemin. The exact nature of the latter linkage in intact cytochrome *c* is at present unknown. The unusual thio-ether structure was inferred from the postulated configuration of porphyrin *c*, a dicysteine adduct of protoporphyrin obtained, under vigorous hydrolysis conditions, from cytochrome *c* (2). The question of the existence of porphyrin *c* preformed in the cytochrome *c* molecule has not been settled to full satisfaction. Theorell had adopted the cautious view that porphyrin *c* could be a preparational artifact (3). Zeile and Meyer (4) have succeeded, however, in preparing porphyrin *c* from cytochrome *c* under conditions claimed by them to exclude the possibility of the creation of dicysteine-porphyrins through the condensation of vinyl and thio groups. With certain reservations Theorell (5) has therefore returned to his original view of the presence of some form of thio-ether linkage in the so called hemin *c* of cytochrome *c*.

It appears that insufficient attention has been directed towards the absorption spectra of cytochrome *c* and its derivatives, as well as the interpretation of these spectra, particularly from the standpoint of the presence of a modified hemin in cytochrome *c*. Absorption constants, upon an iron basis, at characteristic wave-lengths for ferrocytochrome *c* and for the pyridine derivatives of ferroproto-, ferromeso-, and ferrocoproporphyrin have been presented by the writer in a previous communication (6). In this paper data are furnished upon the spectra of cyanide, pyridine, globin, carbon monoxide, and globin carbon monoxide derivatives of the above hemins, as well as upon the spectra of corresponding derivatives prepared from hemoglobin and ferrocytochrome *c*. The assembled evidence now appears to justify the following interpretations. (1) The various spectra fall into several characteristic pattern groups, the shape



and intensity of absorption being determined by the nature of the groups coordinating with the hemin iron. (2) The wave-length location of the  $\alpha$ - and  $\beta$ -bands in the derivatives studied is, on the other hand, largely a function of the hemins themselves, and most probably of the groups substituted in positions 2 and 4 (Fischer's notation (7) applied to iron porphyrins belonging to etioporphyrin Type III). The presence of unmodified vinyl groups in these positions in the protoporphyrin complexes (to which derivatives of protohemin and hemoglobin belong) accounts for an appreciable shift of  $\alpha$ - and  $\beta$ -bands towards the longer wave-lengths in comparison with the location of maxima in the corresponding derivatives of meso- and coprohemin and in cytochrome *c* and its derivatives. From this the inference is drawn that natural cytochrome *c* does not contain an unmodified protohemin with free vinyl groups in its structure.

### Methods

Spectrophotometry was carried out by the technique described in earlier papers (6, 8). The protohemin was prepared from washed erythrocytes of man by the method of Drabkin and Austin (9). The other hemin preparations and the cytochrome *c* were the same upon which certain spectrophotometric constants (6) and iron (10) had been previously determined. The values of  $\epsilon$  ( $c = 1$  mm per liter,  $d = 1$  cm.)<sup>1</sup> are reported upon an Fe basis, 1 mm = 1 mm of Fe. The concentration of individual solutions was determined spectrophotometrically upon aliquots suitably prepared, with the following  $\epsilon$  values at characteristic maxima: 26.1 at 550  $m\mu$  for ferrocytochrome *c* in aqueous solution (6), 9.4 at 536  $m\mu$  for ferricytochrome *c* cyanide in 0.2 M NaOH, 11.5 at 540  $m\mu$  for ferrihemoglobin cyanide (cyanmethemoglobin), 11.3 at 545  $m\mu$  for dicyanide ferriprotoporphyrin (11), 9.7 at 537  $m\mu$  for the cyanide derivative of ferrimesoporphyrin, and 10.7 at 535  $m\mu$  for cyanide ferricoproporphyrin.

The cyanide derivatives of the hemins were prepared by the addition of excess solid KCN to solutions of the respective ferrihemins in 0.200 M NaOH. In those cases in which pyridine was employed, the final concentration of the nitrogenous base was 6.19 M (50 per cent by volume). Reduction, when required, was obtained by means of solid sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ), a high grade reagent (Eimer and Amend, low in iron). Conversion to carbon monoxide derivatives was accomplished in the following way. Approximately 10 cc. of the pigment solution were placed in a small tonometer (capacity 75 cc.). Solid dithionite was then added to the

<sup>1</sup> In the notation  $\epsilon$  ( $c = 1$  mm per liter,  $d = 1$  cm.),  $\epsilon = (1/(c \times d)) \times \log I_0/I$ , where the concentration  $c$  is expressed in mm per liter, the depth  $d$  in cm., the original intensity  $I_0$  is 1.0, and the intensity of transmitted light  $I$  is expressed as a fraction of unity. Hereafter in this manuscript the symbol  $\epsilon$ , defined as above, will be used.

solution, and the air quickly swept out from the tonometer by a stream of illuminating gas, washed through NaOH and water. The solution was equilibrated in the usual manner, with several renewals of the gas and rotation of the tonometer during a period of about 20 minutes. The solution was then admitted into a cuvette of 1 cm. depth used for spectrophotometry, about 2 mg. more of the reductant being added before the cuvette was sealed. This procedure was adopted to insure maintenance of complete reduction both during exposure to CO and subsequent measurement.

The globin used in these experiments was prepared by an acetone-hydrochloric acid method, modified from that of Schenk (12) and of Anson and Mirsky (13); washed human erythrocytes, diluted with 1 volume of distilled water and saturated with CO, were employed as the starting material. The details of the preparation are postponed for later presentation. Sedimentation measurements (14) indicate that globin has a molecular weight of 34,000. In the hemoglobin molecule, two globin units of this molecular weight magnitude would each be associated with 2 iron atoms (2 hemin residues). The equivalent weight of globin, referred to 1 atom of iron, was therefore taken as 17,000. The concentration of globin solutions was based upon their N content, the figure of 16.9 per cent being used for total N (15), and the values are reported in mM, the above equivalent weight of 17,000 being employed as the molecular magnitude of reference. The absorption data presented for the derivatives prepared from the globin have been corrected for the presence of traces of hemin in the protein. Since the globin solutions were made up in 0.2 M NaOH, the derivatives studied are those of *denatured globin*. In describing these derivatives the term *globan* (analogous with the term edestan) will be used in the general nomenclature previously adopted (11). To avoid confusion it should be stated that globan derivatives may be prepared either by the addition of alkali to hemoglobin, or by the addition of hemin to alkaline solutions of globin. Both types of preparations were employed, and they will be distinguished by appropriate description in the text. The pH of the various solutions studied was determined by a glass electrode. In the very alkaline range the values are only approximate, and were measured against 0.1000 M NaOH as a standard, set at pH 13.0.

### Results

The  $\epsilon$  values at characteristic maxima (of  $\alpha$ - and  $\beta$ -bands) and minima for various derivatives of ferrohemin, hemoglobin, and ferrocytochrome *c* are collected in Tables I and II. An examination of the wave-length locations of these maxima and minima indicates clearly that the  $\alpha$ - and  $\beta$ -bands of the derivatives prepared from ferroprotoporphyrin are shifted appreci-

TABLE I

*ε Values for Characteristic Maxima and Minima, with Their Wave-Length Locations, of Various Derivatives of Hemins, Hemoglobin, and Cytochrome c in Alkaline Solutions pH 12 to Greater Than 13\**

Derivative	Wave-length	$\epsilon$	$\frac{\epsilon}{\epsilon_m}$	$\frac{\epsilon}{\epsilon_m}$
	<i>mμ</i>			
Cyanide ferroprotoporphyrin, from protohemin	570 (α)†	10.60	1.17	1.67
	560 (m)	9.05		
	540 (β)	15.12		
Cyanide ferromesoporphyrin, from mesohemin	557 (α)	10.12	1.44	2.42
	550 (m)	7.04		
	528 (β)	17.02		
Cyanide ferrocoproporphyrin, from coprohemin	558 (α)	10.58	1.39	2.28
	550 (m)	7.63		
	528 (β)	17.36		
Reduced cyanide derivative, from dog hemoglobin	568 (α)	10.68	1.15	1.62
	555 (m)	9.26		
	540 (β)	14.99		
Reduced cyanide derivative, from cytochrome c	555 (α)	16.63	2.20	2.06
	545 (m)	7.55		
	527 (β)	15.55		
Pyridine ferroprotoporphyrin from protohemin	558 (α)	30.90†	3.41	1.79
	540 (m)	9.06†		
	525 (β)	16.25†		
Pyridine ferromesoporphyrin, from mesohemin	547 (α)	33.41†	3.73	2.11
	532 (m)	8.95†		
	518 (β)	18.91†		
Pyridine ferrocoproporphyrin, from coprohemin	547 (α)	32.71†	3.52	2.04
	532 (m)	9.29†		
	518 (β)	19.00†		
Reduced pyridine derivative, from dog hemoglobin	558 (α)	29.11	3.13	1.67
	542 (m)	9.31		
	528 (β)	15.58		
Reduced pyridine derivative, from cytochrome c	551 (α)	29.10	3.36	2.15
	539 (m)	8.67		
	522 (β)	18.62		
Globan ferroprotoporphyrin, from human globin and protohemin	558 (α)	30.90§	3.49§	1.58§
	542 (m)	8.85§		
	528 (β)	13.97§		
Globan ferromesoporphyrin, from human globin and mesohemin	548 (α)	28.70	4.20	2.04
	533 (m)	6.84		
	519 (β)	13.93		
Globan ferrocoproporphyrin, from human globin and coprohemin	548 (α)	15.57	2.11	1.56
	530 (m)	7.38		
	518 (β)	11.50		
Globan ferroprotoporphyrin, from dog hemoglobin	558 (α)	26.53	3.55	1.65
	542 (m)	7.48		
	528 (β)	12.36		

TABLE I—*Concluded*

Derivative	Wave-length	$\epsilon$	$\frac{\epsilon_{\alpha}}{\epsilon_m}$	$\frac{\epsilon_{\beta}}{\epsilon_m}$
	<i>m<math>\mu</math></i>			
Globin ferroprotoporphyrin, from hemoglobin of man	558 ( $\alpha$ )	29.10	2.92	1.49
	541 ( <i>m</i> )	9.95		
	528 ( $\beta$ )	14.80		
Ferrocytochrome <i>c</i> in 0.2 M NaOH	550 ( $\alpha$ )	28.38	3.89	2.02
	535 ( <i>m</i> )	7.29		
	520 ( $\beta$ )	14.73		

\* The concentration of NaOH in most of the solutions was 0.2 M.

†  $\alpha$ , maximum of the  $\alpha$ -band;  $\beta$ , maximum of the  $\beta$ -band; *m*, minimum, with a spectral interval of 1.5 to 2  $m\mu$ .

‡ Average values, including corresponding values published previously (6).

§ Corresponding values for globin ferroprotoporphyrin, prepared from dog globin and protohemin, are, at the same wave-lengths, 30.60 ( $\alpha$ ), 9.62 (*m*), and 15.68 ( $\beta$ ), with ratios of 3.18 ( $\epsilon_{\alpha}/\epsilon_m$ ) and 1.63 ( $\epsilon_{\beta}/\epsilon_m$ ).

|| Corresponding values (6) upon ferrocytochrome *c* solutions, pH 4.10 to 4.92, are 26.11 at 550  $m\mu$  ( $\alpha$ ), 7.39 at 535  $m\mu$  (*m*), and 15.47 at 520  $m\mu$  ( $\beta$ ), with ratios of 3.53 ( $\epsilon_{\alpha}/\epsilon_m$ ) and 2.09 ( $\epsilon_{\beta}/\epsilon_m$ ).

ably towards the longer (red) wave-lengths in comparison with corresponding derivatives of ferromeso- and ferrocoproporphyrin. This shift in wave-length location of the spectra of protohemin derivatives is apparently not a function of the group coordinating with the hemin iron, but a property of the hemins themselves. This is brought out by the fact that the characteristic wave-length shift is evident in the cases of derivatives of protohemin whatever the nature of the coordinating group—cyanide, pyridine, globin, carbon monoxide, or carbon monoxide globin.

The wave-length locations of the maxima of the  $\alpha$ - and  $\beta$ -bands of derivatives prepared from hemoglobin (which contains protohemin in its structure) are very similar to corresponding derivatives prepared from protohemin. This was perhaps to be expected of derivatives prepared from hemoglobin in alkaline solution, but the location of the maxima in carbon monoxide hemoglobin, pH 7.4 to 8.0, in which undenatured globin is coordinated with the iron, is also in the wave-length regions characteristic of carbonyl derivatives of ferroprotoporphyrin (Table II). On the other hand, the  $\alpha$  and  $\beta$  maxima of ferrocytochrome *c*, at pH 4.1 to 4.9 (foot-note to Table I), ferrocytochrome *c* at pH 13 (Table I), and the cyanide, pyridine, and carbonyl derivatives of the latter (Tables I and II) are located in the wave-length regions characteristic of the derivatives of ferromeso- and ferrocoproporphyrin, and are very considerably displaced from the regions associated with protohemin derivatives. Small but consistently reproducible differences in  $\epsilon$  values (Table I and its foot-note) were found for

ferrocytochrome *c* in 0.2 M NaOH and for the same ferrocytochrome *c*, prepared by the method of Keilin and Hartree (16) and dissolved in water. The latter solutions are usually slightly acid (6). It is uncertain whether

TABLE II

*ε* Values for Characteristic Maxima and Minima, with Their Wave-Length Locations, of Carbonyl (CO) Derivatives of Hemins, Hemoglobin, and Cytochrome *c* in Alkaline Solutions

Derivative	Wave-length <i>mμ</i>	<i>ε</i>	$\frac{\epsilon_{\alpha}}{\epsilon_m}$	$\frac{\epsilon_{\beta}}{\epsilon_m}$
Carbonyl ferroprotoporphyrin, from protohem, pH 13+*	573 ( $\alpha$ )†	15.10	1.19	1.15
	558 ( <i>m</i> )	12.67		
	543 ( $\beta$ )	14.57		
Carbonyl ferromesoporphyrin, from mesohemin, pH 13+	562 ( $\alpha$ )	15.43	1.38	1.34
	550 ( <i>m</i> )	11.17		
	532 ( $\beta$ )	14.96		
Carbonyl ferrocoproporphyrin, from coprohem, pH 12.1	557 ( $\alpha$ )	10.57	1.16	1.20
	540 ( <i>m</i> )	9.14		
	527 ( $\beta$ )	10.93		
Carbonyl globin ferroprotoporphyrin, from human globin and protohem, pH 13+	571 ( $\alpha$ )	12.52	1.14	1.17
	557 ( <i>m</i> )	10.93		
	542 ( $\beta$ )	12.83		
Carbonyl globin ferromesoporphyrin, from human globin and mesohemin, pH 13+	559 ( $\alpha$ )	12.52	1.21	1.24
	548 ( <i>m</i> )	10.32		
	533 ( $\beta$ )	12.85		
Carbonyl globin ferrocoproporphyrin, from human globin and coprohem, pH 11.8	559 ( $\alpha$ )	10.54	1.21	1.23
	545 ( <i>m</i> )	8.70		
	529 ( $\beta$ )	10.67		
Carbonyl globin ferroprotoporphyrin, from dog hemoglobin, pH 13+	571 ( $\alpha$ )	11.81	1.09	1.15
	557 ( <i>m</i> )	10.80		
	542 ( $\beta$ )	12.42		
Carbonyl ferrohemoglobin (carbon monoxide hemoglobin), from dog hemoglobin, pH 7.4 to 8.0 ( <i>cf.</i> (8))	569 ( $\alpha$ )	14.39	1.23	1.26
	555 ( <i>m</i> )	11.70		
	539 ( $\beta$ )	14.77		
Carbonyl ferrocytochrome <i>c</i> , pH 12.3	550 ( $\alpha$ )	16.90	1.68	1.26
	539 ( <i>m</i> )	10.03		
	523 ( $\beta$ )	12.68		

\* pH 13+ indicates pH greater than 13.

†  $\alpha$ , maximum of the  $\alpha$ -band;  $\beta$ , maximum of the  $\beta$ -band; *m*, minimum, with a spectral interval of 1.5 to 2 *mμ*.

the differences in extinction may be ascribed to slight denaturation of the cytochrome *c* protein in alkaline solution (1, 5). However, this consideration does not disturb present interpretations of data, since the wave-length locations of the maxima remain unaltered with change in pH.

Figs. 1 to 4 illustrate the influence which the group coordinating with the hemin iron has upon the shape and intensity of absorption. In Fig. 1 the absorption spectrum curves of the cyanide, pyridine, and carbon monoxide derivatives of ferromesoporphyrin are utilized to typify three distinct

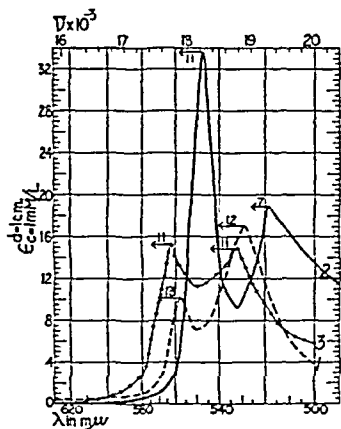


FIG. 1

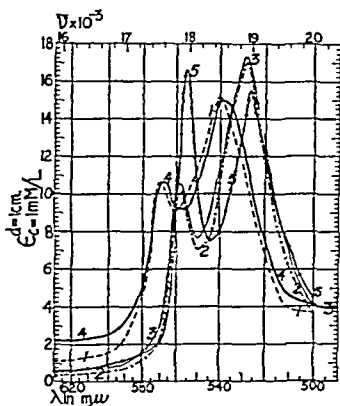


FIG. 2

FIG. 1. Patterns of light absorption, exemplified by derivatives of ferromesoporphyrin. Curve 1, cyanide ferromesoporphyrin, representative of Pattern Group 1; Curve 2, pyridine ferromesoporphyrin, representative of Pattern Group 2; Curve 3, carbonyl ferromesoporphyrin, representative of Pattern Group 3. Concentration of hemin Fe, 0.082 mM per liter in the case of the pyridine derivative, 0.162 mM per liter for the cyanide and carbonyl derivatives. In all cases, NaOH concentration, 0.2 M;  $\text{Na}_2\text{S}_2\text{O}_4$  concentration, approximately 5 mM per liter. Pyridine concentration, 6.19 M; cyanide concentration, approximately 400 mM per liter. The horizontal arrows and appended numbers represent the magnitude in mμ of the shift of maxima towards longer wave-lengths in corresponding derivatives of ferroprotoporphyrin.

FIG. 2. Absorption spectra of cyanide derivatives of ferrohemins, hemoglobin, and ferrocytochrome *c*. Pattern Group 1. Curve 1, cyanide ferroprotoporphyrin; Curve 2, cyanide ferromesoporphyrin; Curve 3, cyanide ferrocoproporphyrin; Curve 4, the reduced cyanide derivative prepared from dog hemoglobin in alkaline solution, probably cyanide ferroprotoporphyrin; Curve 5, the reduced cyanide derivative prepared from cytochrome *c* in alkaline solution, ferrocytochrome *c* cyanide. The concentration of total pigment varied from 0.0992 to 0.1733 mM per liter. In all cases, NaOH concentration, 0.2 M; cyanide concentration, approximately 400 mM per liter;  $\text{Na}_2\text{S}_2\text{O}_4$  concentration, approximately 5 mM per liter.

light absorption patterns into which the spectra of the various derivatives fall. Pattern Groups 1, 2, and 3 are characterized respectively by the spectra of the cyanide, pyridine (or globan), and carbonyl (or carbonyl globan) complexes. Fig. 1 also indicates the magnitude of shift towards

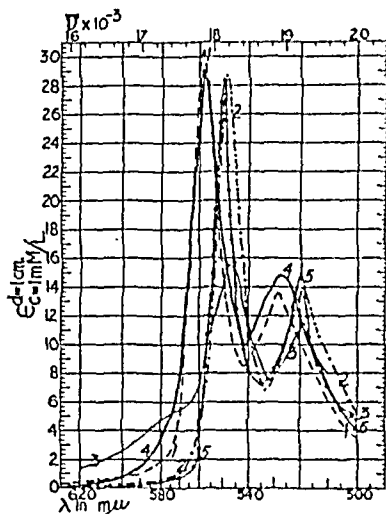


FIG. 3

FIG. 3. Absorption spectra of globan (denatured globin) derivatives of ferrohemins and the spectra of hemoglobin and ferrocytochrome *c* in alkaline solutions. Pattern Group 2. Curve 1, globan ferroprotoporphyrin, prepared from human globin and protohemin. Concentration of hemin Fe, 0.0659 mm per liter; concentration of globin, 0.0447 mm per liter, referred to an equivalent weight of 17,000. Curve 2, globan ferromesoporphyrin, prepared from human globin and mesohemin. Concentration of hemin Fe, 0.082 mm per liter; concentration of globin, 0.0447 mm per liter, referred to an equivalent weight of 17,000. Curve 3, globan ferrocoproporphyrin, prepared from human globin and coprohemin. Concentration of hemin Fe, 0.0377 mm per liter; concentration of globin, 0.765 mm per liter, referred to an equivalent weight of 17,000. Curve 4, globan ferroprotoporphyrin, prepared from hemoglobin of man. Concentration of hemoglobin, 0.0935 mm per liter, referred to an equivalent weight of 16,700 (containing 1 Fe atom). Curve 5, ferrocytochrome *c* in alkaline solution. Concentration of cytochrome *c*, 0.0772 mm per liter, upon an iron basis (6). In all cases, concentration of NaOH, 0.2 M; concentration of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, approximately 5 mm per liter.

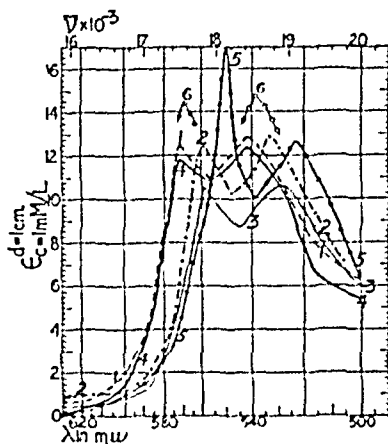


FIG. 4

FIG. 4. Absorption spectra of carbonyl globan derivatives of ferrohemins and of carbonyl derivatives of hemoglobin and ferrocytochrome *c* in alkaline solutions. Pattern Group 3. Curve 1, carbonyl globan ferroprotoporphyrin, prepared from protohemin. Concentration of hemin Fe, 0.0659 mm per liter; concentration of globin, 0.447 mm per liter, referred to an equivalent weight of 17,000. Curve 2, carbonyl globan ferromesoporphyrin, prepared from mesohemin. Concentration of hemin Fe, 0.082 mm per liter; concentration of globin, 0.447 mm per liter, referred to an equivalent weight of 17,000. Curve 3, carbonyl globan ferrocoproporphyrin, prepared from coprohemin. Concentration of hemin Fe, 0.0874 mm per liter; concentration of globin, 0.637 mm per liter, referred to an equivalent weight of 17,000. Curve 4, carbonyl globan ferroprotoporphyrin, prepared from dog hemoglobin. Concentration of hemoglobin, 0.0655 mm per liter, referred to an equivalent weight of 16,700 (containing 1 Fe atom). Curve 5, carbonyl ferrocytochrome *c*, prepared from cytochrome *c* in alkaline solution. Concentration of cytochrome *c*, 0.0625 mm per liter, upon an iron basis (6). Curve 6, incompleted curve showing the maxima of the spectrum of carbonyl ferrohemoglobin (carbon monoxide hemoglobin), pH 7.4 to 8.0 (8). In all cases, except Curve 6, concentration of NaOH, 0.2 M; concentration of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, approximately 5 mm per liter.

the longer wave-lengths (of the order of  $11\text{ m}\mu$ ) of the maxima of corresponding derivatives of ferroprotoporphyrin. As Figs. 2 to 4 show, the wave-length shift is, of course, not limited to the maxima, but is a displacement of practically the whole absorption curve.

The spectra of different cyanide (Pattern Group 1), globan (Pattern Group 2), and carbonyl globan (Pattern Group 3) derivatives are presented in Figs. 2, 3, and 4. The presentation of the spectra of individual pyridine and carbonyl complexes (representatives are shown in Fig. 1) was considered unnecessary. The absorption patterns of the pyridine derivatives belong unmistakably to the same group as the globan, while those of the carbonyl derivatives are of the same general shape as the spectra of the carbonyl globan complexes. These similarities of pattern are suggested by a comparison of the ratios of extinction at maxima to extinction at minimum given in Tables I and II. The globan derivatives of ferrohemins may be considered perhaps to bear a closer structural similarity than do the pyridine derivatives to ferrocytochrome *c*. The spectrum of ferrocytochrome *c* in alkaline solution is found actually to have a closer similarity to the spectrum of globan ferromesoporphyrin than to that of pyridine ferromesoporphyrin (Figs. 1 and 3).

While the spectra of the cyanide and pyridine derivatives of ferrocoproporphyrin proved to be virtually identical with the spectra of the corresponding derivatives of ferromesoporphyrin (Table I), this was not the case with globan ferrocoproporphyrin (Table I and Fig. 3). Qualitatively the spectrum of the latter derivative is indubitably that of a reduced hemochromogen and belongs to Pattern Group 2, but it is apparent that the  $\epsilon$  values are appreciably lower than those for the other globan complexes studied, and there is evidence of greater absorption in the red spectral region. Within the limits of variation in concentration of coprohemin and globin which could be tested, 1 and 10 cm. cuvettes being used for spectrophotometry, the spectrum remained essentially unaltered. The explanation for the aberrant spectrum of globan ferrocoproporphyrin must remain in abeyance.

Attention needs to be directed to several further points. As the legend to Fig. 3 indicates, globan ferroproto- and globan ferromesoporphyrin were obtained from the respective hemins under conditions in which the globin concentration in mM (referred to an equivalent weight of 17,000) was lower than the concentration in mM of hemin Fe. That the reaction of globan with the ferrohemins is complete appears to be satisfactorily assured by the fact that alteration in spectrum could not be accomplished either by moderately lowering the concentration of the hemins or raising the concentration of the globin, as well as by comparison with the  $\epsilon$  values of globan ferroprotoporphyrin, prepared from hemoglobin (Table I and Fig. 3).



These findings indicate that globan possesses an extraordinarily great affinity (in comparison with simpler nitrogenous bases) for ferroproto- and ferromesohemins, and are in this regard in agreement with studies published by Zeile and Gnant (17), although the extinction coefficients given by the latter are lower than those reported here. A detailed study of the equilibria involved in the reaction of ferroprotoporphyrin with several different proteins has been made by the writer. This investigation will be presented separately.

The spectra of cyanide, pyridine, globan, and carbonyl derivatives prepared from ferroprotohemins are practically identical with the spectra of corresponding derivatives prepared from hemoglobin in alkaline solution. This finding may be taken tentatively as presumptive evidence that the two sets of preparations result in identical complexes.

The shift of absorption towards the longer wave-lengths observed in the spectra of protohemins is characteristic not only of the ferro complexes (to which the presentation of data has been limited), but appears to be a general property evident also in the ferri derivatives of protohemins. An example is the spectra of the cyanide ferri complexes, with a single maximum in the green spectral region. The  $\epsilon$  values and their wave-length locations of these derivatives have been furnished under "Methods." It is seen that the maximum of cyanide ferriprotoporphyrin and of cyanide ferrihemoglobin is displaced characteristically in comparison with the maximum of cyanide ferrimesoporphyrin and of cyanide ferricoproporphyrin. Also, as in the case of the ferro derivatives, the maximum of ferri cytochrome *c* cyanide falls in the spectral region characteristic of the corresponding ferrimeso and ferricopro compounds. It may be added that in alkaline solutions of similar pH the spectrum of protohemins itself is displaced towards the red in comparison with the spectra of ferrimeso- and ferricoprohemins.

#### DISCUSSION

Ferrocyanochrome *c*, carbon monoxide hemoglobin, and pyridine ferroprotoporphyrin have been found in Pauling's laboratory (1, 18) to be diamagnetic. From this the deduction was drawn that the constitution of these substances is that of hexacoordination complexes of the octahedral covalent type (analogous in configuration with the ferrocyanide ion). It appears probable that all the derivatives of ferrohemins examined here belong structurally to the same class. Nevertheless, the nature of the group coordinating with the hemin iron plays a dominant rôle in determining the shape and intensity of the absorption pattern in the visible spectral region. It is sufficient to comment that the three different structures, cyanide ion, neutral nitrogenous base (such as pyridine), and carbon monox-

ide, each place a characteristic stamp upon the visible spectrum. In this class of derivatives (excluding other types of configuration, such as the essentially ionic (18)) only these three patterns of absorption have been uncovered. It is all the more remarkable that the distinctive differences in light absorption are confined to the  $\alpha$ - and  $\beta$ -bands; the rest of the complex absorption pattern involving several other maxima and minima is practically identical for the above cyanide, pyridine, and carbonyl complexes, as shown by the writer's analysis of their complete visible and ultraviolet spectra, published in preliminary form (19). Further discussion of this point may be postponed except to state that the analysis (19) disclosed the unique character of the  $\alpha$ - and  $\beta$ -bands.

The finding (and its general applicability) that the spectra of protohemin and its various derivatives are shifted appreciably towards the longer (red) wave-lengths in comparison with the spectra of corresponding derivatives of meso- and coprohemin appears of real interest. Until stronger arguments are forthcoming in support of more subtle reasons for this phenomenon, it seems best to relate it to structural differences in the hemins themselves. The only obvious structural difference is the presence in protohemin of vinyl residues in positions 2 and 4 of the tetrapyrrolic ring, whereas meso- and coprohemin contain respectively ethyl and propionic acid groups in these positions. The latter groups exert presumably no special effect upon the spectrum, since the maxima of the spectra of corresponding derivatives of meso- and coprohemin are at virtually identical wave-lengths. It would seem therefore that the primary difference in configuration, somehow spectroscopically operative, may be the unsaturated character of the vinyl ( $-\text{CH}=\text{CH}_2$ ) group. Comprehensive studies, which are of pertinent interest, have been carried out by Hausser and Kuhn and their collaborators upon the analysis of the spectra of homologous series of synthetic and naturally occurring polyene dyes of the type  $\text{R}-(\text{CH}=\text{CH})_n-\text{R}'$ . These studies, published in summary (20) and in detail (21) following Hausser's death, establish the rôle of the conjugated double bond in the shift of spectra towards longer wave-lengths, the magnitude of the shift being a non-linear function of  $n$ . While the study of Hausser, Kuhn, and Seitz (22) upon mesoporphyrin is not pertinent in this connection, it seems probable to the writer that the wave-length shift observed in the present work may also be correlated with an increase in conjugated double bonds in the protohemin due to the presence of vinyl radicals in addition to the conjugated double bond system of the porphine nucleus itself, common to the different hemins studied.

The fact that the  $\alpha$ - and  $\beta$ -bands of ferrocytochrome *c* are located at wave-lengths close to those of the maxima of pyridine ferromesoporphyrin had been observed earlier (6, 23), but no structural implication had been

connected with this circumstance. The deduction that ferrocytochrome *c* does not contain in its structure an unmodified protohemin with free vinyl groups follows logically from the observations which have been recorded and from the interpretation which has been applied. Any modification in the vinyl groups which would nullify their double bond would be expected to produce the spectroscopic characteristics which have been found. Theorell's thio-ether linkage (5) satisfies this condition. The wave-length location of its visible absorption spectrum becomes therefore in itself the most direct evidence which has been furnished for the presence of a modified hemin in natural cytochrome *c*.

Acknowledgment is due to Miss H. Lorraine Leidy for helpful assistance. The writer is indebted also to Dr. Curt Porter and Dr. W. Mansfield Clark respectively for small but valuable samples of synthetic coprohemin and mesohemin used in this work.

#### SUMMARY

The absorption spectra of various derivatives of ferroprotoporphyrin, ferromesoporphyrin, ferrocoproporphyrin, hemoglobin, and ferrocytochrome *c* have been studied.

The maxima of the  $\alpha$ - and  $\beta$ -bands of the spectra of ferromeso- and ferrocoprohemin derivatives are at practically identical wave-lengths, while the spectra of corresponding derivatives of ferroprotohemin are shifted appreciably towards the longer (red) wave-lengths. The maxima of the spectra of ferrocytochrome *c* and its derivatives are located at wave-lengths characteristic of the ferromeso- and ferrocoproporphyrin compounds.

The spectra of the derivatives of the ferrohemins fall into three distinct pattern groups, characteristic respectively of the cyanide, pyridine (or denatured globin), and carbonyl (or carbonyl denatured globin) complexes.

The following interpretations have been applied. (1) The nature of the group coordinating with the hemin iron determines the intensity and shape of the absorption pattern in the visible spectral region ( $\alpha$ - and  $\beta$ -bands). (2) The wave-length location of the maxima is a function of the hemins themselves. The shift of absorption towards longer wave-lengths in the case of protohemin and its derivatives is ascribed to the double bond in the vinyl groups present in this hemin. This view represents an extension to a new class of substances of the findings of Hausser and Kuhn (20, 21) upon the influence of conjugated double bonds on the spectra of polyene derivatives.

The deduction is drawn that natural cytochrome *c* does not contain an unmodified protohemin with free vinyl groups in its structure.

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# IDENTIFICATION OF THE CARBOHYDRATE GROUP IN THE NICOTINAMIDE NUCLEOTIDES

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The structural formula of codehydrogenase I (cozymase) which was proposed in 1936 as a working hypothesis (1) has been found to be correct in all details by subsequent work. The only remaining question of major importance was the nature of the pentose in the nicotinamide nucleotide part of the molecule.

In the present paper, experiments are reported which show that this pentose is *d*-ribose. This finding also has significance with respect to the structural formula of codehydrogenase II, since the reversible transformation of codehydrogenase I into codehydrogenase II has been established experimentally (2).

The carbohydrate was isolated in the following manner. Enzymatic splitting of cozymase yielded the nicotinamide nucleoside, consisting of nicotinamide and pentose, from which the carbohydrate was liberated by acid hydrolysis. After removal of nicotinamide the pentose could be isolated and identified by its properties and by preparation of the *p*-bromophenylhydrazone.<sup>1</sup>

A reinvestigation of the nature of the pentosephosphoric acid from cozymase was also undertaken, in order to confirm the previous claims concerning the position of the phosphoric acid in the pentose phosphate. Earlier experiments along this line had been carried out with cozymase preparations containing some impurities (5), and the resulting product was not well characterized. With pure cozymase as a starting material for

<sup>1</sup> After this paper had been submitted for publication, a paper by von Euler, Karrer, and Usteri (3), dealing with the carbohydrate of cozymase, came to the author's attention. Von Euler and coworkers claim the same result as communicated here. The experimental data, however, as presented in their paper, are not strictly convincing. The only product isolated is a pentose phenylosazone. *d*-Arabinose and *d*-ribose give identical phenylosazones. The method of isolation yields a mixture of the 2 pentose molecules of cozymase. Therefore, only a yield of more than 50 per cent would be significant for the pentose of the nicotinamide nucleotide part, since the pentose of the adenylic acid part has been recognized previously (4) to be *d*-ribose. Starting from 1.0 gm. of cozymase, von Euler and coworkers have isolated 24 mg. of a pentose phenylosazone, corresponding to 11.0 mg. of pentose, which is 2.4 per cent of the theoretical amount. (Note added September 21, 1942.)

the preparation of the pentosephosphoric acid, it was demonstrated that the product obtained was identical in all respects with *d*-ribose-5-phosphoric acid as described by Levene and coworkers (4). Thus, the previous findings (6) have been confirmed.

#### EXPERIMENTAL

*Preparation of Codehydrogenase I*—Codehydrogenase I was prepared from yeast<sup>2</sup> according to the method described previously (7), with some modifications. The phosphotungstic acid precipitation was found to be unnecessary provided a good quality of yeast was used as a starting material. The purification steps were carried out in the following order: heat extraction of yeast, lead acetate precipitation of impurities, barium hydroxide treatment to remove impurities, successive precipitation of cozymase as mercuric, silver, and cuprous salts, and finally alcohol precipitation. From several batches of yeast (50 kilos) somewhat more than 10 gm. of about 75 per cent pure cozymase were obtained. By subsequent fractionation as barium and lead salts a main fraction (3.4 gm.) of at least 95 per cent purity was obtained, which served as starting material, partly for the preparation of nicotinamide nucleoside, and partly for the isolation of pentosephosphoric acid. The properties of this codehydrogenase I preparation are apparent from the following data.

*Elementary Analysis*<sup>3</sup>— $C_{21}H_{27}O_{14}N_7P_2 \cdot 21H_2O$  (699.2)

Calculated. C 36.04, H 4.46, N 14.01, P 8.87

Found. " 35.79, " 4.23, " 13.53, " 8.76

*Pentose Determination*—Bial's reaction as modified by Mejbaum (8) was used; pentose calculated, 42.9 per cent; found, 42.2. In this and in many other quantitative determinations reported here the phosphoric acid content of the preparation was taken as a basis for calculation to account for the moisture. Ribose, xylose, and arabinose give identical results when used as standards. The method was found to be almost as accurate as a colorimetric phosphate determination. The samples were heated 30 minutes instead of 20 minutes as recommended by Mejbaum. A Klett-Summerson photoelectric colorimeter was used for the readings.

*Titration with Alkali*—Phenolphthalein was used as indicator; 0.020 mm of codehydrogenase I required 1.05 ml. of 0.02  $\times$  NaOH (0.021 mm).

*Catalytic Hydrogenation*—Palladium catalyst (9) was used; calculated, 101 c.mm. of  $H_2$  per mg. (mol. wt., 663); found, 104 c.mm. of  $H_2$ , 97.5 c.mm. of  $H_2$  per mg.

<sup>2</sup> Obtained from Anheuser-Busch, Inc., St. Louis, to whom the author wishes to express his sincerest thanks.

<sup>3</sup> Microanalyses reported in this paper were carried out partly by Dr. C. Tiedcke, New York, partly by C. H. Spence and R. H. Morris, Galveston.

*Coenzyme Activity*—The coenzyme activity as tested by the fermentation method (10) was maximal as compared with a standard preparation.

*Ultraviolet Absorption of Dihydro Derivative*—For determination of the ultraviolet absorption spectrum a Hilger quartz spectrograph (model 498) was used. The preparation of the dihydro derivative was carried out as given by Warburg (9); found,  $\epsilon_{340\text{ m}\mu} = 5.5 \times 10^3$ . Warburg has reported  $\epsilon_{340\text{ m}\mu} = 5.9 \times 10^3$ ; Ohlmeyer (11),  $\epsilon_{340\text{ m}\mu} = 6.3 \times 10^3$ ; and Schlenk and Günther (7),  $\epsilon_{340\text{ m}\mu} = 5.6 \times 10^3$  ( $\epsilon = 1/(l \times c) \log I_0/I$ ;  $l = \text{cm.}$ ;  $c = \text{moles per liter}$ ).

*Preparation of Nicotinamide Nucleoside*—2.35 gm. of cozymase were split enzymatically (12), the phosphatase from almond press-cake<sup>4</sup> discovered by Bredereck (13) being used. The isolation of the nucleoside was carried out as described previously: removal of the phosphoric acid split off by  $\text{Ba}^{++}$ , dialysis, removal of adenosine by  $\text{Ag}^+$ , precipitation of the nucleoside by phosphotungstic acid, and decomposition of the precipitate by alcohol-ether mixture. The subsequent treatment with picric acid as recommended earlier was omitted, since it was found to involve a considerable loss of material. By precipitation with alcohol and ether 720 mg. of nucleoside as sulfate were obtained. Spectrophotometric determination of the dihydro derivative showed the preparation to be 75 per cent pure. For further purification 712 mg. were dissolved in methyl alcohol and precipitated in two fractions by ether. The main fraction consisted of 410 mg. of nucleoside of >90 per cent purity. The ratio of pentose to nicotinamide (12) was found to be 1:1.02. Spectrophotometric determination of the dihydro derivative gave  $\epsilon_{340\text{ m}\mu} = 6.3 \times 10^3$ ; phosphate determination, <0.05 per cent P.

*Hydrolysis of Nicotinamide Nucleoside*—400 mg. of nicotinamide nucleoside were dissolved in 25 ml. of 0.1 N sulfuric acid and hydrolyzed 120 minutes at 100°. After cooling, sulfuric acid was removed quantitatively by barium hydroxide. The solution was concentrated to 3 ml. and an equal volume of 10 per cent gold chloride solution was added to precipitate nicotinamide and nicotinic acid, the latter having been formed by hydrolysis of nicotinamide. After 24 hours at 0° the precipitate was filtered off and washed with 1 ml. of water. The solution was extracted with ether in a liquid extraction apparatus to remove the excess of gold chloride. When the solution was colorless, it was treated for the removal of traces of gold and chloride in the following manner: silver sulfate solution was added as long as a precipitate was formed; after centrifugation, a slight excess of  $\text{Ag}^+$  was removed by hydrogen sulfide treatment, and, after filtering, the solution was brought to a small volume; sulfate was removed

<sup>4</sup> Obtained from the American Almond Products Company, Glendale, California, to whom the author wishes to extend his gratitude.



by treatment with barium hydroxide, an excess being avoided. The remaining solution was evaporated to a sirup, the crystallization of which involved considerable difficulties. The product obtained was not free from ash. Yield, 118 mg.; m.p. 85°.

*Elementary Analysis*— $C_5H_{10}O_5$  (150.1)

Calculated. C 40.00, H 6.72

Found. " 40.19, " 6.75 (ash subtracted)

*Polarimetric Determination*—For the polarimetric determination the concentration of the solution was determined by the quantitative pentose test (8). Observed,  $[\alpha]_D^{20} = -20.6^\circ$  ( $c = 0.800$  in  $H_2O$ ). The specific rotation reported in the literature for *d*-ribose is  $-19.5^\circ$  to  $-21.5^\circ$  (4).

*Preparation of p-Bromophenylhydrazone*—No difficulties were encountered in preparing the *p*-bromophenylhydrazone. For this purpose 70 mg. of the sugar were dissolved in 1.5 ml. of absolute alcohol, treated with an equimolar amount of *p*-bromophenylhydrazine, and allowed to stand for 48 hours at 30°. The resulting product was recrystallized from 2 ml. of absolute alcohol. Yield, 83 mg.; m.p. 165–166°.

*Elementary Analysis*— $C_{11}H_{13}O_4N_2Br$  (319.2)

Calculated. C 41.35, H 4.73, N 8.77, Br 25.03

Found. " 41.02, " 5.11, " 9.00, " 25.06

*Polarimetric Determination*— $[\alpha]_D^{25} = +5.8^\circ$  ( $c = 1.46$  in absolute alcohol). The specific rotation reported in the literature for *d*-ribose-*p*-bromophenylhydrazone is  $+5.7^\circ$  (4).

*Isolation of Pentosephosphoric Acid*—670 mg. of cozymase were hydrolyzed by 50 ml. of 0.1 *N* sulfuric acid at 100° for 4 hours. Adenine and nicotinamide are split off quantitatively under these conditions, while 20 per cent of the total phosphoric acid is liberated. Adenine was removed as the silver salt and the isolation of pentosephosphoric acid as barium salt was carried out according to Levene and Jacobs (14). After repeated precipitation by alcohol 465 mg. of a product were obtained which had the following properties.

*Elementary Analysis*— $C_5H_8O_8P\text{Ba}$  (365.5)

Calculated. C 16.45, H 2.46, P 8.48, Ba 37.59

Found. " 16.70, " 2.54, " 8.24, " 37.25

*Polarimetric Determination*— $[\alpha]_D^{25} = +6.5^\circ$  ( $c = 2.90$  in  $H_2O$ ). Levene and Stiller (15) have reported  $[\alpha]_D^{20} = +5.99^\circ$  for the barium salt of *d*-ribose-5-phosphoric acid. For polarimetric determination of the free acid  $Ba^{++}$  was removed by an excess of sulfuric acid. Found,  $[\alpha]_D^{25} = +17.2^\circ$  ( $c = 1.05$  in 0.02 *N*  $H_2SO_4$ ). Levene gives  $[\alpha]_D^{20} = +16.09^\circ$  for the natural compound, and  $[\alpha]_D^{20} = +16.54^\circ$  for the synthetic compound (15).

*Periodic Acid Test*—To ascertain the position of the phosphate group in the pentosephosphoric acid the product was treated with periodic acid

TABLE I  
Formation of Formaldehyde from Pentose and Pentosephosphoric Acid by Periodic Acid

Compound examined	Amount examined	Formaldehyde obtained*	Mole per cent
	$\gamma$ moles	$\gamma$ moles	
d-Ribose.....	38.8	39.0	101
".....	77.6	76.0	98
d-Ribose-3-phosphoric acid.....	33.2	14.0	42
".....	66.4	27.7	41.7
d-Ribose-5-phosphoric ".....	53.1	2.8	5.3
".....	70.8	2.9	4.1
Pentosephosphoric acid from cozymase.....	90.0	6.1	6.8
".....	112.3	6.0	5.4

\* Formaldehyde was determined by precipitation with dimethyldihydroresorcinol (dimedon).

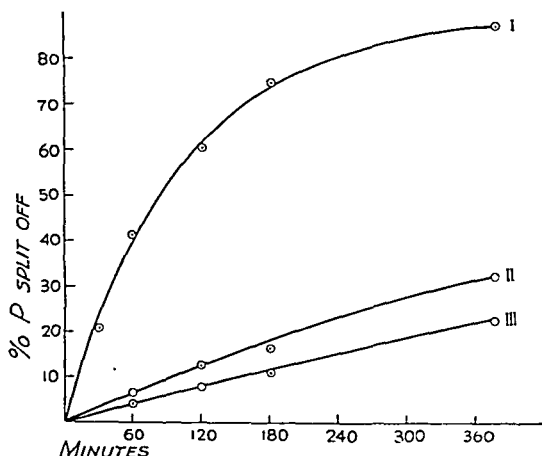


FIG. 1. Rate of liberation of phosphoric acid from (Curve I) adenosine-3'-phosphoric acid, (Curve II) codehydrogenase I, and (Curve III) adenosine-5'-phosphoric acid in 0.1 N  $H_2SO_4$  at  $100^\circ$ .

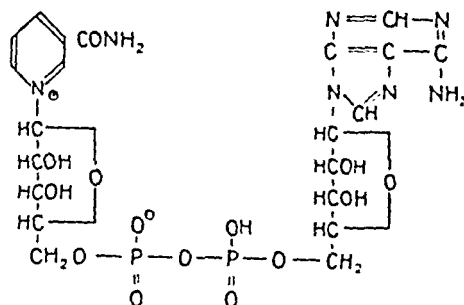
according to Fleury and Lange's method (16), as improved by Reeves (17). Compounds with a primary alcoholic group adjacent to a secondary alcoholic group ( $-CHOH \cdot CH_2OH$ ) yield formaldehyde if treated with periodic

acid. If the primary alcoholic group is occupied by a phosphoric acid radical, very little formaldehyde is formed. Von Euler, Karrer, and Becker (6) and later Kiessling and Meyerhof (18) have used this method to determine the position of the phosphoric acid in the pentose phosphates from several nucleotides. Table I shows the results of the examination of the ribosephosphoric acid from cozymase as compared with some other compounds.

*Acid Hydrolysis*—The rate of splitting of phosphoric acid from codehydrogenase I, adenosine-5'-phosphoric acid, and adenosine-3'-phosphoric acid, as shown in Fig. 1, is also in agreement with the assumption that in codehydrogenase I both phosphoric acid radicals are linked to carbon atom 5 of the pentose molecules.

### DISCUSSION

The results of the experiments reported leave no doubt that the carbohydrate group of the nicotinamide nucleotide moiety in codehydrogenase I (cozymase) is identical with *d*-ribose. The earlier investigations indicating that the phosphoric acid groups of cozymase are linked to carbon atoms 5 of the pentose molecules were confirmed.



I. Codehydrogenase I (cozymase)

It is believed that the identification of the carbohydrate group of the nicotinamide nucleotide is the last step of major importance in establishing the structure of codehydrogenase I (see Formula I) as proposed in 1936 (1). This structural formula is based on the following results: empirical formula,  $C_{21}H_{27}O_{14}N_7P_2$ , and titration (19); isolation of the basic components, adenine (10) and nicotinamide (20); isolation of pentosephosphoric acid (5) and its identification as *d*-ribose-5-phosphoric acid; isolation of adenosine diphosphate (21), the structure of which is well established by earlier work of Lohmann (22), Embden and Schmidt (23), Levene (4), and Gulland and Holiday (24); experiments concerning the linkage of nicotinamide to the carbohydrate, which were initiated by Warburg (25) and successfully con-

cluded by Karrer (26); isolation of the nicotinamide nucleoside (12) and identification of its carbohydrate group as *d*-ribose.

Thus, the structure of codehydrogenase I seems now to be well established.

#### SUMMARY

1. Nicotinamide nucleoside was prepared from codehydrogenase I. Its carbohydrate group was found to be *d*-ribose.

2. Both ribosephosphoric acid molecules of codehydrogenase I have the phosphoric acid radical linked to carbon atom 5 of the pentoses.

3. Some facts pertaining to the structure of codehydrogenase I are briefly discussed.

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# STUDIES ON PITUITARY LACTOGENIC HORMONE

## VII. A METHOD OF ISOLATION\*

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The methods which are generally accepted for the preparation of highly purified lactogenic hormone from either sheep or ox pituitaries are the procedures described by Lyons (1) and by Bates and Riddle (2). The acidic acetone extraction of pituitaries devised by Lyons has been demonstrated not only in this laboratory but also by White, Bonsnes, and Long (3) as the most suitable starting material for the isolation of the lactogenic hormone. With the same starting extract, a new method is described in the present paper for the isolation of the hormone in pure form. The hormone is found to behave identically in biological and physicochemical characteristics with that prepared by Lyons and appears also to be the same protein isolated by White, Bonsnes, and Long.

### *Methods of Assay*

Both the crop sac weight method of Riddle, Bates, and Dykshorn (4) and the minimum crop stimulation method of McShan and Turner (5) have been applied to the estimation of activity of lactogenic preparations. The results reported here are based on the minimal response. The Silver King strain of pigeons was used at  $33 \pm 3$  days of age. A daily dose of 0.5 cc. was injected intramuscularly for a period of 4 days, followed by autopsy 96 hours after the first injection. The crop sacs were examined for the degree of proliferation and weighed. The extent of the reaction was classified on the basis of gross observation as falling into one of four arbitrary groups, Grade 1 being the minimal detectable response as seen by transmitted light after the muscular coat was stripped, the maximum reaction accompanied by abundant crop milk formation being classified as a Grade 4 reaction. The minimal reaction is not accompanied by perceptible weight increase of the crop (1 to 2 gm.); the maximum reaction gives 2 to 4 gm. crops.

In order to interpret the potency of preparations in terms of international

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units (I.U.), a sample of the international standard of lactogenic hormone has been injected into six groups of pigeons, the routine technique being used. It was found that 5 I.U. gave the minimum stimulation (Grade 1 reaction), whereas 40 I.U. caused a marked increase in crop sac weight (weight, 2.6 gm.). Since most of the pure preparations give minimal crop sac stimulation in a total dose of 0.15 to 0.20 mg. it may be said that 1 mg. of the lactogenic hormone whose preparation is described here contains at least 25 I.U.

### *Methods of Isolation*

1. 2 kilos of fresh sheep glands were ground twice in an electric meat grinder with 1 liter of water. The ground gland was then poured into 8 liters of acid-acetone solution.<sup>1</sup> After the mixture had been stirred for 1 hour, it was filtered by suction. The residue was washed in the filter with 1 liter of 80 per cent acetone. The procedure was carried out at room temperature; the succeeding steps were performed in a cold room at 2-3°.

2. To the clear brown filtrate and washings, 32 liters of chilled acetone (-5°) were added. The mixture was allowed to settle overnight. The supernatant was siphoned off and the precipitate was washed two or three times with cold acetone on a suction filter. The precipitate, designated as the acid-acetone powder, served as the starting material for further purification. It may be stored in a desiccator until needed. From 2 kilos of sheep pituitaries, about 35 gm. of acid-acetone powder were obtained.

3. The acid-acetone powder was next extracted with 1 liter of 0.10 M  $\text{Na}_2\text{HPO}_4$  and centrifuged; as some floating material remained after prolonged centrifugation of this first extract, it was filtered after centrifugation. The residue was reextracted twice with 600 cc. of the same solvent. The whole extraction procedure took about 36 hours. The residue showed no adrenotropic or lactogenic activities and was therefore discarded.

4. The combined extracts were then brought to half saturation of  $(\text{NH}_4)_2\text{SO}_4$  by the addition of solid salt. The mixture was allowed to stand for 4 hours and then centrifuged. The supernatant was devoid of hormonal activities and was discarded. The precipitate was then dissolved in about 250 cc. of water and dialyzed<sup>2</sup> until salt-free.

5. The dialyzed material was then made to pH 3.0<sup>3</sup> with 1 M HCl. After dilution to 1 per cent solution, a saturated NaCl solution was added dropwise with mechanical stirring, until the concentration of NaCl became

<sup>1</sup> The acid-acetone was prepared by mixing 8 liters of acetone with 200 cc. of concentrated HCl (c.p. grade; sp. gr. 1.19).

<sup>2</sup> Dialysis against running tap water (15-16°) for 6 hours was followed by dialysis against 3 liters of distilled water at 2-3° for 15 hours.

<sup>3</sup> pH measurements were made with a Beckman glass electrode.

0.36 M. It was then centrifuged and the supernatant saved for the purification of the adrenotropic hormone.

6. The 0.36 M NaCl precipitate was dissolved in water with the aid of a few drops of 1 M NaOH until the pH of the solution became approximately 7.5. A small amount of precipitate, which remained undissolved, was removed by centrifuging. The clear supernatant was then diluted with distilled water to about 1 per cent solution and dialyzed against a phosphate buffer<sup>4</sup> of pH 6.4 for 24 hours. The insoluble material, pH 6.3, was then removed by centrifugation. It contained approximately 5 i.u. per mg. of protein and was discarded.

7. The supernatant was then dialyzed against an acetate buffer<sup>5</sup> of pH 5.6 for 24 hours. After being centrifuged, the supernatant had little protein nitrogen and was discarded.

8. The pH 5.6 precipitate was dissolved in about 1 per cent solution with the aid of a few drops of 1 M HCl and made pH 3.0. Steps (5), (6), and (7) were repeated twice.

9. The final pH 5.6 precipitate was again dissolved in acid solution; the concentration of the protein was about 1 per cent. The solution was then dialyzed against an acetate buffer<sup>6</sup> at pH 4.0. There appeared some precipitate which had about 7 i.u. per mg. of protein and was discarded.

10. The pH 4.0 solution was dialyzed against the buffer of pH 5.6 and steps (7) and (9) were repeated twice.

11. The final pH 4.0 solution was made pH 3.0 with 1 M HCl and step (5) was repeated until the 0.36 M NaCl supernatant contained a constant nitrogen value per cc. in two successive 0.36 M NaCl precipitations.

The final NaCl precipitate contained a lactogenic potency of 25 to 30 i.u. per mg. of protein. It was shown to behave as a single substance with respect to solubility, electrophoresis, and diffusion.

### *Homogeneity Studies*

*Electrophoresis*—The materials obtained in each step outlined above were examined in a Tiselius electrophoresis apparatus, the Longworth scanning method being used for the estimation of homogeneity and migration. Most of the experiments were conducted in a buffer of pH 7.0. A few were done in a buffer of pH 4.0.

<sup>4</sup> The buffer was prepared by dissolving 2.464 gm. of  $\text{KH}_2\text{PO}_4$ , 2.840 gm. of  $\text{Na}_2\text{HPO}_4$ , and 58.50 gm. of NaCl in 2 liters of solution.

<sup>5</sup> The buffer was prepared by mixing 360 cc. of 1 M NaOH and 200 cc. of 0.2 M acetic acid and diluted to 2 liters.

<sup>6</sup> The buffer was prepared by mixing 1666 cc. of 1 M acetic acid and 334 cc. of 1 M NaOH.



The first precipitate<sup>7</sup> (step (7)), pH 5.6, *i.e.* the material obtained before the dialysis at pH 4.0, was always found to have two components in the electrophoresis experiment; the fast moving one constituted about 20 per cent of the total protein. The average mobilities of these two components were  $6.02 \times 10^{-6}$  and  $3.61 \times 10^{-6}$  sq. cm. per volt per second in phosphate buffer of pH 7.0 and ionic strength 0.10 at 1.5°.

After this material was purified by repeated dialysis in the acetate buffer of pH 4.0 (step (9)), the soluble protein was shown to migrate essentially as a single substance in electrophoretic experiments and had a mobility<sup>8</sup> of  $3.37 \times 10^{-6}$  sq. cm. per volt per second in the phosphate buffer, pH 7.0.

*Diffusion*—By the use of a Northrop and Anson sintered glass diffusion disk (6), it was possible to examine biological homogeneity of the lactogenic preparations after various periods of diffusion.<sup>9</sup> Before introduction into the diffusion cell, the 1.2 per cent solution of the hormone was dialyzed at room temperature for at least 24 hours against 2 liters of phosphate buffer,<sup>10</sup> pH 7.23, of ionic strength 0.1. During the process of diffusion, the buffer solution in the outside chamber was changed every 24 hours for a period of 6 days and samples were assayed for lactogenic activity in squabs. Differences in the various samples as regards the crop sac reaction were not observed. The preparation was hence homogeneous in its diffusion behavior.

*Solubility*—Two lactogenic preparations were subjected to solubility tests (7). It was found that solutions to which the protein had been added in amounts which were 10 times that necessary for saturation underwent no change in the amount of dissolved material (25°). The solvent used was 0.357 M NaCl of pH 2.25. These tests therefore also confirm the homogeneity of the preparation.

#### SUMMARY

A method is described for the isolation of pituitary lactogenic hormone from sheep or ox glands. The preparation is found to be chemically pure by electrophoretic, solubility, and diffusion tests and has an activity of 25 to 30 I.U. per mg.

<sup>7</sup> The biological activity of this precipitate was not different from that of the pure hormone. This could, of course, be the case if the contamination were an *inert* protein, as less than 20 per cent of an inert contaminant cannot be detected by the biological method.

<sup>8</sup> Thus it may be concluded that the slow moving component in the precipitate of step (7) is the lactogenic hormone. Further support of this conclusion is obtained by the isolation of the fast moving component by use of the compensation device of Longsworth. The fast moving material thus obtained was devoid of lactogenic activity when injected into pigeons at a total dose of 1.0 mg.

<sup>9</sup> The results of these determinations will be presented in Paper VIII.

<sup>10</sup> Butanol was added to 2 per cent to prevent bacterial growth.

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# STUDIES ON PITUITARY LACTOGENIC HORMONE

## VIII. DIFFUSION AND VISCOSITY MEASUREMENTS\*

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In Paper VI of this series (1) we have determined the molecular weight of the pituitary lactogenic hormone from osmotic pressure measurements and analytical data and found it to be 26,000. Recently White, Bonsnes, and Long (2) reported the molecular weight of the hormone to be 32,000 or higher, as calculated from ultracentrifugal experiments. The difference in these two values is not surprising, for many experiments<sup>1</sup> have shown that molecular weight determinations as obtained by osmotic pressure or by ultracentrifugation methods need not be in close agreement. It would seem that a decision as to the correct molecular weight for the hormone would be aided by additional methods of study. In this paper the molecular size and shape of the lactogenic hormone are examined in the light of viscosity and diffusion data. The importance of the molecular shape in relation to the biological activity of the hormone will be discussed in a later paper.

### EXPERIMENTAL

*Concentration Determination*—Semimicro-Kjeldahl nitrogen analyses were used for the determination of protein concentration with the factor 100/15.8 for conversion into protein values. A series of solutions with increasing concentrations of the pure protein was made and the optical rotation determined in a Bellingham-Stanley polarimeter with a sodium light source. From such results, the hormone concentration in any solution could be estimated from the optical rotation data.

*Viscosity*—Viscosity measurements<sup>2</sup> were performed in an Ostwald viscometer described by Neurath, Cooper, and Erickson (4). A working volume of 25 cc. was generally employed. The time of flow, which was measured with a high precision stop-watch, was determined with an ac-

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<sup>1</sup> See Table I of Oncley's paper (3).

<sup>2</sup> Carolyn F. White assisted in these determinations.

curacy of  $\pm 0.05$  second. At least six measurements were carried out for each experiment. A water bath at  $25^\circ$  was used.

Most of the experiments were done in a phosphate buffer of pH 7.00 of ionic strength 0.1 containing 2 per cent butanol.<sup>3</sup> The protein solution must be dialyzed for at least 24 hours against the buffer before the viscosity determinations.

*Density*—Density determinations were made in a pycnometer of 9 cc. volume at  $25^\circ$ .

*Diffusion*—The membrane method of Northrop and Anson (5) was used to determine the diffusion coefficient of the hormone. The sintered glass diffusion cell<sup>4</sup> had a volume of about 45 cc. and was clamped rigidly, by means of a slot and clasp arrangement, in a metal frame which was mounted firmly in a constant temperature room of  $25^\circ \pm 0.1^\circ$ . The protein solution was dialyzed against the buffer until no appreciable difference in conductivity was found between the protein and buffer solutions. The amount of buffer used outside of the membrane was 25 cc. and it was replaced with a fresh solution every 24 hours during the run. The quantity of protein nitrogen which diffused was determined by Kjeldahl analysis.

The calibration of the diffusion membrane was made with 2.0 M NaCl and 0.1 M KCl. The diffusion coefficients of NaCl (6) and KCl (7) were taken to be 1.27 and 1.46 sq. cm. per day, at  $25^\circ$  respectively.

*Materials*—The lactogenic hormone preparations were made according to the method described in Paper VII of this series (8). One beef hormone preparation (No. L-383B2) was kindly supplied by Dr. W. R. Lyons. All preparations were found electrophoretically homogeneous in the Tiselius apparatus. Preparation L-1165 was also shown to be homogeneous in solubility studies.

### Results

*Specific Rotation*—By means of the data summarized in Table I a plot of the observed rotation against the concentration of the hormone is found to be a straight line. The concentration of the hormone can thus easily be secured by the determination of the optical rotation. Since from Table I, 1.0 per cent solution has an observed angle of  $0.9^\circ$ , the specific rotation of the sheep lactogenic hormone is  $-40.5^\circ$  at  $25^\circ$ .

The optical rotation of the hormones isolated from beef glands was also determined and its specific rotation is identical with that for the sheep hormone.

<sup>3</sup> Butanol was added to prevent bacterial growth.

<sup>4</sup> We are greatly indebted to Dr. John H. Northrop for a loan of his diffusion cell to make our experiments possible.

*Partial Specific Volume*—This quantity may be calculated by the equation,

$$V_1 = \frac{1}{\rho} \left[ 1 - \frac{1 - W_1}{\rho} \frac{d\rho}{dW_1} \right] \quad (1)$$

which is modified from that derived by Kraemer (9). The partial volume,  $V_1$ , of a protein can thus be obtained by knowing the density,  $\rho$ , of the solution containing different weight fractions,  $W_1$ , of the protein. From results shown in Table I,  $d\rho/dW_1$  equals 0.280. The partial specific volume of the hormone can hence be computed from Equation 1 and is found to be 0.721.

TABLE I

*Optical Rotations, Densities, and Relative Viscosities of Sheep Pituitary Lactogenic Hormone Solutions at 25° in Phosphate Buffer of pH 7.00 and Ionic Strength 0.10*

Preparation No.	Concentration, protein per liter of solution	Optical rotation	Density	Relative viscosity, $\frac{\eta}{\eta_0} - 1$	$\left(\frac{\eta}{\eta_0} - 1\right) \frac{1000}{cV_1}$
	gm.	degree			
L-1165S	2.8		1.00129	0.014	6.94
	3.9		1.00170	0.020	7.10
	5.5	0.50	1.00216	0.025	6.30
	7.9	0.71	1.00281	0.038	6.68
L-1180G	4.3	0.36	1.00191	0.019	6.13
L-1183BS	1.03	0.93	1.00352	0.050	6.73
L-1184H	8.7		1.00301	0.041	6.55
	4.3		1.00188	0.021	6.77
L-1185H	3.5*	0.31	1.00252	0.017	6.74
L-1197H	4.5	0.39	1.00185	0.021	6.47
L-383B†	8.5		1.00296	0.041	6.70
Average.....					6.65

\* The solvent for this experiment was acetate buffer, pH 4.05.

† A beef pituitary hormone prepared by Dr. W. R. Lyons.

*Viscosity*—The results of viscosity measurements are given in Table I. It may be noted that six sheep and one beef preparations were employed. In one case (Preparation L-1185H) the solvent used was an acetate buffer, pH 4.05, of ionic strength 0.10, while other measurements were made in a phosphate buffer, pH 7.00. A straight line relationship exists between the viscosity and the protein concentration at least up to 1.0 per cent solution. It can be seen that the viscosity of the beef hormone is essentially the same as that of the sheep preparations.

*Diffusion*—In the membrane diffusion method of Northrop and Anson, the diffusion coefficient,  $D$ , is calculated by the equation,

$$D = KQ_{cc.}/t \quad (2)$$

where  $Q_{cc.}$  is the number of cc. of the concentrated solution that contains the amount of the substances diffused, in the time,  $t$ , where  $K$  is a cell constant. The  $K$  of our cell was found to be 0.125. In Table II the results of the diffusion experiments are given.

Since the sintered glass disk method for determination of the diffusion constant involves the selection of a primary standard, the constant obtained by Lamm's free diffusion technique (10) is generally considered to be

TABLE II

*Diffusion Constant of Pituitary Lactogenic Hormone at 25° Obtained in Sintered Glass Cell*

pH	Buffer*	Concentration <i>per cent</i>	$Q_{cc.}$ per day	$D$	
				$sq. \text{ cm. per day} \times 10^{-7}$	$sq. \text{ cm. per sec.} \times 10^{-7}$
3.42	Acetate	0.91	0.620	7.75	8.95
6.30	Phosphate	0.52	0.635	7.91	9.20
7.00	"	0.26	0.640	8.00	9.26
7.23	"	1.18	0.615	7.70	8.90
Average.....				7.85	9.08

\* All buffers have ionic strength 0.10 and contain 2 per cent butanol to prevent bacterial growth.

more accurate. Mehl (11) has made a careful comparison of the diffusion constants secured by these two methods and arrived at a conclusion that the free diffusion value is about 11 per cent higher than that obtained by the sintered glass disk method. Using this correction together with that for the viscosity of the solvent, we have computed the diffusion constant of the hormone to be  $10.78 \times 10^{-7}$  sq. cm. per second at 25°. When this value is reduced to 20°, the value becomes  $9.0 \times 10^{-7}$  sq. cm. per second.

#### DISCUSSION

According to Einstein, the volume-concentration and the specific viscosity should have the following relationship,

$$\left( \frac{\eta}{\eta_0} - 1 \right) \frac{1000}{cV_1} = \Phi \quad (3)$$

with  $\Phi = 2.5$ , provided the molecule is strictly spherical. If  $\Phi$  is greater than 2.5, the molecule may be an ellipsoidal shape. From the results in

Table I, it is evident that the lactogenic hormone molecule is far from spherical<sup>6</sup> and we can assume that it is a prolate ellipsoid. According to the equation of Simha (12), the ratio of the long to the short axis of a prolate ellipsoid,  $b/a$ , has a value of 5.7. If Polson's empirical viscosity equation (13) is utilized,  $b/a$  becomes 5.2, but, since the Simha equation has been shown to be applicable to protein molecules of low asymmetry (3, 4), the value  $b/a = 5.7$  was chosen for these calculations.

The shape of a protein molecule may also be expressed by the frictional constant,  $f/f_0$ , which can also be computed if we know the ratio of the long to the short axis of a prolate ellipsoid of revolution. Thus, with the aid of Perrin's equation (14), the frictional constant  $f/f_0$  of the lactogenic hormone is found to be 1.29.

TABLE III  
*Molecular Size and Shape of Pituitary Lactogenic Hormone*

Molecular weight	
Osmotic pressure.....	26,500
Analytical data.....	25,000
Diffusion and viscosity.....	22,000
Sedimentation-diffusion*.....	32,000
Diffusion constant ( $D_{20w}$ )	
Sintered glass membrane.....	$9.0 \times 10^{-7}$
Free diffusion*.....	$7.5 \times 10^{-7}$
Partial specific volume ( $V_1$ ).....	0.721
Viscosity coefficient ( $(\frac{\eta}{\eta_0} - 1)1000/cV_1$ ).....	6.65
Dissymmetry constant ( $f/f_0$ )	
Viscosity data.....	1.29
Sedimentation-diffusion†.....	1.37

\* Reported by White, Bonsnes, and Long (2).

† Calculated from the data of White, Bonsnes, and Long (2).

Finally it should be possible to calculate the molecular weights by utilizing the diffusion constant, partial specific volume, and the frictional constant,

$$M = \frac{R^3 T^3}{162 \pi^2 \eta_0^3 N^2} \frac{(f_0/f)^3}{D^3 V_1} \quad (4)$$

<sup>6</sup> In this first approximation, we have purposely neglected the influence of hydration. It has been pointed out by Neurath, Cooper, and Erickson (4) that a protein with  $b/a = 4.9$ , like serum albumin, at zero hydration, assumes a spherical shape provided the molecule is 96 per cent hydrated. Since most proteins are about 33 per cent hydrated, it is reasonable to assume that the lactogenic hormone is correspondingly hydrated. On this assumption, the ratio of  $b/a$  of the ellipsoid is hardly reduced to unity and it may therefore be concluded that the hormone is not of spherical shape.



where  $T$  is the temperature,  $\eta_0$  the viscosity of the solvent, and  $N$  Avogadro's number. The molecular weight of the hormone calculated by this equation is 22,000. It may be recalled that the molecular weight obtained from osmotic pressure measurements is 26,000 (1). Owing to the disadvantage of unavoidable magnification of the possible experimental errors in the diffusion and viscosity data in Equation 4, the agreement of these two values would seem satisfactory.

White, Bonsnes, and Long (2) reported the molecular weight of their homogeneous preparation of lactogenic hormone as 32,000 with the sedimentation constant,  $S_{20} = 2.65 \times 10^{-13}$ , and the diffusion constant,  $D_{20} = 7.5 \times 10^{-7}$ . From these two values, the frictional constant is calculated to be 1.37, which suggests that the hormone is even more asymmetric than the viscosity data indicate.

Table III will serve to summarize the data bearing on the molecular size and shape of the anterior hypophyseal lactogenic hormone.

#### SUMMARY

1. The optical rotation of the lactogenic hormone has been found to have a linear relation to the concentration; the specific rotation is  $-40.5^\circ$ .
2. The partial specific volume of the hormone is shown to be 0.721.
3. The diffusion constant,  $D_{20}$ , has been determined by the sintered glass membrane method and found to be  $9.0 \times 10^{-7}$  sq. cm. per second.
4. From viscosity data, the dissymmetry constant of the hormone is 1.29.
5. With the diffusion constant and dissymmetry constant as herein determined, the molecular weight of the hormone is computed to be 22,000.

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# THE EFFECTS OF A MAGNESIUM-DEFICIENT DIET ON THE SERUM PHOSPHATASE ACTIVITY IN THE ALBINO RAT\*

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In view of the activating effect of magnesium on phosphatase, first observed by Erdtman (1), and the possibility that magnesium may otherwise affect phosphatase activity (1-4), it appeared to us that a change in the enzyme activity should be a primary result of magnesium deprivation, or a secondary result induced by impaired nutrition.

In 1932, Kruse, Orent, and McCollum (5) demonstrated, conclusively, that magnesium is an essential element for certain bodily activities, growth, and life. These authors succeeded in preparing a diet containing only 0.18 mg. per cent of magnesium, but otherwise adequate, which they fed to young rats. For the first time they were able to give a graphic description of the spectacular series of symptoms which are characteristic of severe magnesium deficiency. The investigations of Tufts and Greenberg (6), who studied in rats the physical and chemical changes resulting from varying levels of dietary magnesium, afford a further insight into various degrees of magnesium deficiency. Certain observations made by these two groups of investigators (5, 6) are pertinent to the consideration of the manner in which magnesium deprivation affects phosphatase activity.

Orent, Kruse, and McCollum (7) reported a decrease in magnesium from 2.96 to 0.81 mg. per cent in the pooled blood serum samples from young rats, weighing 35 to 45 gm., which had received the magnesium-poor diet for 5 days. During the course of magnesium deficiency impaired nutrition was seldom observed (5). Throughout an experimental period of 33 days the growth curves for the magnesium-deficient rats were found to be fair but not optimum. Anorexia and inanition, according to these authors, did not play a part in this decrease in the growth rate. The fact that their magnesium-deficient animals died before loss of weight occurred was regarded as particularly significant.

In young dogs which had received the magnesium-low diet and in which the symptoms and sequelae of magnesium deficiency developed less rapidly than in the rat, Kruse, Orent, and McCollum (8) observed a marked dis-

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turbance in the blood lipids shortly after the serum magnesium reached the low level of 0.4 to 0.9 mg. per cent. This disturbance in the blood lipids was interpreted as evidence of a failure in the fat metabolism.

Tufts and Greenberg (6), who employed diets containing 0.4 to 5.0 mg. per cent of magnesium, were able to observe that the course of magnesium deficiency in the rat consisted of two fairly definite phases. Like Kruse, Orent, and McCollum (5), they observed that the prominent early symptoms of the deficiency are vasodilatation and hyperemia of the exposed skin areas. These symptoms were considered to be the chief manifestations of the first phase.

Tufts and Greenberg (6) state that the development of hyperexcitability, the beginning of nutritive failure, and the beginning of a rise in plasma magnesium apparently occur simultaneously, and mark the end of the first and the onset of the second phase of magnesium deficiency. The second phase was characterized as the period in which the development of malnutrition, cachexia, and kidney damage occur. These authors found little reduction of growth in the first phase of the deficiency, but a marked reduction in the second phase. On a diet containing 1.5 mg. per cent of magnesium the period of good growth was found to last 2 to 3 weeks, when a rapid decline in the rate of growth ensued, which sometimes led to a loss in weight.

Both groups of authors (5, 6) observed profound disturbances in the metabolism of calcium, magnesium, and phosphorus. Orent, Kruse, and McCollum (7) report that as early as the 5th day on the deficient diet, and thereafter for the duration of the survival period, the bones of their experimental animals yielded more ash than did those of the controls. The increased weight of the bones was found to be due in large measure to the excessive deposition of calcium during the first 5 days on the diet.

Our data show that in the magnesium-deficient rat a marked decrease in the serum phosphatase activity occurs parallel with, or immediately following, a drop in the serum magnesium to subnormal levels, and well before the signs of impaired nutrition are evident.

### *Technique and Methods*

The experimental diet was prepared according to the directions of Kruse, Orent, and McCollum (5). For the control diet, sufficient magnesium sulfate was included in the diet to give a magnesium content of approximately 53.9 mg. per cent. These diets were prepared in 2 kilo portions, and kept in closed glass containers in a refrigerator. The stock diet, from which the animals were transferred to the special diets, was a commercial preparation known as Purina fox chow.

Young albino rats, weighing 47 to 118 gm., were restricted to the mag-

nesium-low diet, and litter mates of these animals were placed on the control diet, or allowed to remain on the stock diet. The amount of food and distilled water allotted daily to the animals was sufficient to allow consumption *ad libitum*.

At the time intervals shown in Table I, the animals were placed under light ether anesthesia and bled by cardiac puncture. Sufficient blood serum was obtained for the estimation of individual serum inorganic phosphorus and phosphatase values, but in some instances it was necessary to determine calcium and magnesium on blended samples.

Estimations of serum phosphatase activity were made by the Bodansky method (9). Stock chemical solutions, used in this method, were prepared in small quantities at frequent intervals and stored in a refrigerator. The suitability of working solutions, prepared from the stock reagents, was determined before each estimation of phosphorus. Measurements of the phosphatase activity in the blood sera of stock, control, and experimental animals were carried out simultaneously, immediately after the serum samples were obtained. For the determination of phosphatase activity, 3 ml. of an aqueous solution of  $\beta$ -glycerophosphate, buffered to pH 8.6, were incubated with 0.3 ml. of serum for 1 hour at 37°. Inorganic phosphorus was determined on 0.5 ml. samples of serum.

The phosphatase values reported in Table I were determined without added magnesium. Several estimations of phosphatase activity in the pooled blood sera of controls and of experimental animals with added magnesium ions in concentrations of 0.001 to 0.01 *M* revealed no significant change in the phosphatase activity ratio of the two serums. Thus there appeared to be an actual decrease in the phosphatase content of the serum of the magnesium-deficient animal.

Calcium was determined on 1 to 2 ml. of serum by the Kramer and Tisdall method (10) as modified by Tweedy and Koch (11). Serum magnesium was determined by Hoffman's method (12). In most instances the amounts of magnesium in the blood serum of the magnesium-deficient animals were too small for direct estimation by the ordinary colorimeter. In some instances in which an attempt was made to determine the quantities directly, the values are reported as being lower than 1 mg. per cent. In others, a known amount of magnesium was added to the calcium- and protein-free filtrate, and the apparent magnesium value determined by difference.

### Results

With the exception of the animals which were on the magnesium-low diet for 2 or 3 days (Table I), all other animals restricted to this ration exhibited the symptoms of vasodilatation and hyperemia during the course

TABLE I  
*Effect of Magnesium Deprivation on Calcium, Inorganic Phosphorus, and Magnesium Content, and Alkaline Phosphatase Activity of Serum of Young White Rats*

Initial age	Litter No.	Sex distribution	Time on diet	Gain in weight			Serum calcium			Serum inorganic phosphorus			Serum magnesium			Serum phosphatase		
				S*	C†	D‡	S*	C†	D‡	S*	C†	D‡	S*	C†	D‡	S*	C†	D‡
days			days	gm.	gm.	gm.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	units per cent §	units per cent §	units per cent §
28	21	3 ♀	2	8	0		8.0			11.2	11.0	8.5			2.1	52.6	69.2	70.3
28	21	2 ♂	3		7			8.0			10.0	9.5			2.1		53.4	74.6
28	18	3 ♂	4	16	11	12	9.8	8.8	9.3			9.2			0.8	73.0	71.8	46.4
28	18	3 ♂	5	18	20	13	9.8	8.8	9.3			8.3			0.8	62.8	49.1	22.5
28	19	3 ♂	6	30	21	23	10.5	9.3	9.8			8.5			1.0	64.3	52.5	28.9
28	19	3 ♀	9	35	30	23	9.0	9.4	8.8			9.1			0.6	64.3	53.3	19.2
28	18	3 ♀	14	43	32	25	9.0	8.2				8.1				49.2	43.8	47.7
28	18	1 ♀	16	50	61	35	11.0	10.2	11.3			8.7			0.4	45.3	43.8	25.8
28	19	2 ♂																
25	22	1 ♂	5			14			9.9			8.8			2.1			23.7
25	22	1 ♀	7			17			10.7			8.5			0.4			16.6
34	17	3 ♂	11	53	47	43	10.1	10.6	10.7			8.5			<1.0	87.3	58.4	30.2
34	17	1 ♀, 1 ♂	13	27	21	21	9.0					9.1			<1.0	63.2		32.7
48	16	3 ♂	9	37	44	49		9.4	9.3			7.5			<1.0	75.8	34.6	23.9
48	16	2 ♂	10		47	27		10.7	10.6			7.8			<1.0		55.4	29.2
48	16	3 ♀	12	41	38	20	9.0	8.0	9.2			9.1			<1.0	61.1	53.0	38.8
48	16	1 ♀	13		30			8.0										
39	27	3 ♂	20	54	58	44	10.9	11.4	11.5			9.1			1.1	49.0	26.8	15.7
39	27	1 ♀	22			27			10.9			8.0			2.1			25.6
39	29	3 ♀	25	46	48	36	9.6	10.1	10.7			7.5				46.7	18.7	29.9
39	30	1 ♀	26		45			10.6				8.0					31.6	
39	27	1 ♀	26		33			10.9				7.9					39.7	

39	29	3 ♀	27	49	45	30	11.4	11.2	11.1	7.8	7.4	6.2	2.8	2.7	1.2	54.8	45.2	29.6
39	29	1 ♂, 1 ♀	27	79	61		11.2	11.2	12.7		9.2	9.0		2.2	0.8		43.4	24.2
36	30	1 ♂, 2 ♀	26	107	45	36	10.2	10.6	10.8	9.1	8.0	9.2		2.6	0.3	60.1	31.6	27.6
36	30	3 ♂	29	99	97	55	10.9			9.1	10.5	11.3	3.4	2.1	2.4	52.6	40.7	29.8
Range.....																		
Mean.....																		
s.d.....																		
Difference between means.....																		
Standard error of difference between means.....																		
Mean difference divided by its standard error (critical ratio).....																		
							0.1	0.5	0.1	1.1	0.2	1.4	11.4	16.3				
							0.36	0.36	0.39	0.37	0.22	0.19	4.09	4.36				
							0.28	1.39	0.26	3.00	0.91	7.00	2.78	3.74				

The figures enclosed in a brace indicate pooled samples taken from two similarly treated animals.

\* Animals maintained on the stock (fox chow) diet.

† Animals fed the control diet (magnesium-low ration with 0.0539 per cent of magnesium added, in the form of magnesium sulfate).

‡ Animals fed the magnesium-low diet.

§ Bodansky units per 100 ml. of serum.

|| Samples taken from animals during the period of vasodilatation.

of the experiment. These symptoms were first shown by the 28 day-old animals on the 4th day of the low magnesium regimen, but were not exhibited by the 48 day-old animals until the 9th day.

Symptoms of hyperexcitability were usually shown by the 25 to 39 day-old animals while they were still in the hyperemic period. As the animals became increasingly excitable, convulsions were elicited in some of them, but chemical data on these are not included here. During the last week of the experimental period, the animals began to show loss of hair, particularly around the eyes and on the under surfaces of the jaws and neck. The change in the curvature of the nails, reported by Kruse, Orent, and McCollum (5) after a longer experimental period, was not observed by us.

After the 5th day of the experimental period, the gain in weight of animals on the control diet was comparable to that of animals on the stock diet. The animals on the magnesium-deficient diet, however, showed a slower gain in weight than did their controls. In this connection Tufts and Greenberg (6) state that 70 to 75 per cent of the difference in weight gained by their magnesium-deficient rats and the controls could be accounted for by a decreased food intake. Although the consumption of food by our experimental animals was less than that of the controls, the daily intake remained fairly constant after the first 2 days on the diet.

The serum calcium and inorganic phosphorus of the control animals show no significant variation from the values shown by the animals on the stock diet, but there is a slight shift in the Ca:P ratio in the serum of magnesium-deficient animals toward a higher calcium and a lower phosphorus level. In the case of the serum magnesium, however, there is a marked difference between the values exhibited by the experimental animals and those seen in their controls. After a brief period of adjustment to the magnesium-low diet, a rapid decrease occurred in the serum magnesium of the experimental animals. By the 4th or 5th day, the serum magnesium of animals started on the diet at 28 days of age had been reduced to the low level of 0.8 mg. per cent. One animal in this group had a serum magnesium of 0.6 mg. per cent on the 9th day, and another a serum magnesium of 0.4 mg. per cent on the 16th day of the low magnesium regimen. By the 9th day, animals started on the diet at 48 days of age had entered the hyperemic period and showed low serum magnesium and phosphatase values.

At the time the serum magnesium was rapidly decreasing, a slightly slower decrease in the serum phosphatase activity began. Three animals which were restricted to the magnesium-low diet for 2, 3, or 4 days had serum phosphatase activities of 70.3, 74.6, and 46.4 units, respectively (Table I). Although the latter value is about half that shown on the same day by litter mates on the stock and control diets, it is still within

the normal range seen in the animals on the stock diet. The phosphatase activity of 22.5 units, shown by the magnesium-deficient rat on the 5th day, however, is definitely below the normal range seen in the animals on the stock diet, and less than the lowest value encountered in the control series. With the exception of the phosphatase activity of 47.7 units, shown by an experimental animal on the 14th day of the low magnesium regimen, no other instance was encountered in which the phosphatase activity of an experimental animal exceeded that of its control.

The possibility that an increase in the unsaturated fatty acid content of the magnesium-low diet would influence the serum phosphatase activity of the magnesium-deficient rat was suggested by the experiments reported by Weil and Russell (13). From an investigation of the effects of the resorption and utilization of carbohydrate, protein, and lipid fractions of Purina dog chow, and various lipid derivatives, they reached the conclusion that certain unsaturated fatty acids are the dietary factors influencing plasma phosphatase activity in the rat. For obtaining the highest enzyme activity they found a diet containing 8 per cent of lard to be highly effective.

The following experiment was carried out to determine whether an increase in the unsaturated fatty acid fraction of the diet by the substitution of lard for butter would increase the serum phosphatase activity of the magnesium-deficient rat. Ten young male rats, average weight 150 gm., were placed on the unaltered magnesium-low diet, which contained 8 per cent of butter. After 12 days, five of these animals were transferred to a magnesium-low diet differing from the first in that it contained 8 per cent of lard instead of 8 per cent of butter. 6 days later the five animals which had been allowed to remain on the unaltered diet showed an average serum phosphatase activity of 32.5 units and an average serum magnesium of 1.36 mg. per cent. The five animals which had been transferred to the diet containing lard showed an average serum phosphatase of 25.7 units and an average serum magnesium of 1.85 mg. per cent. The average gain in weight of the animals transferred to the diet containing lard was slightly greater than that of the animals which remained on the unaltered diet. These data show that an increase in the unsaturated fatty acid fraction of the diet was ineffective in elevating the serum phosphatase activity of the magnesium-deficient rat.

Both the stock diet and the control diet presumably contained sufficient magnesium for all of the bodily requirements; yet a marked difference is noted in the serum phosphatase values of the animals on these two rations. The results reported by Weil and Russell (13) suggest that the stock diet contained a larger fraction of the unsaturated fatty acids affecting serum phosphatase activity.



## DISCUSSION

When rats (Table I) 28 days of age were restricted to a magnesium-deficient diet, their serum magnesium began to decrease, and by the 4th or 5th day reached the subnormal level of 0.8 mg. per cent. As the serum magnesium approached its lowest normal level, apparently a decrease in the serum phosphatase activity was initiated. By the 5th day, when the serum magnesium had fallen to 0.8 mg. per cent, the serum phosphatase had diminished to 22.5 units. Although less pronounced in some instances, this depression in serum phosphatase activity did not appear to be transitory, since it was observed consistently in experimental rats of different ages at various intervals between 4 and 30 days.

Our observations, particularly with respect to the 28 day-old rats, indicate that we were dealing with a degree of magnesium deficiency very similar to that described by Orent, Kruse, and McCollum (7) in rats 25 days of age. From a study of the mineral composition of the bones of control and magnesium-deficient rats, they discerned that concurrent with the rapid decrease in the serum magnesium of the latter the magnesium entering the bones increased. The increase was slight, since retardation in the usual rate was evident by the 5th day on the diet, and by the 30th day the absolute amount of magnesium was less than half that in the controls. During the first 5 days on the magnesium-deficient diet, calcification of the bones of their experimental animals was greatly increased, but thereafter the rate of calcification was comparable to that seen in the controls. These authors suggest that the abrupt return to a normal rate of calcification may have been due to the onset of puberty or to nutritive failure, similar to that seen in their magnesium-deficient dogs. The effects of magnesium deprivation on the osseous system are explained on the general ground of the close relationship which the metabolism of calcium, magnesium, and phosphorus bears to bone structure.

Although the bones of our animals were not analyzed for their mineral content, the foregoing observations suggest that the decrease in the serum phosphatase activity was accompanied or immediately followed by a brief period of increasing calcification of the bones. That increasing calcification is accompanied by a fall in plasma phosphatase activity has been observed by several investigators. Kay (14) has shown that in certain generalized diseases of bone, such as osteitis fibrosa, osteomalacia, and rickets, in which there is poor, or faulty, calcification, there is a high plasma phosphatase activity. In certain cases of generalized osteitis fibrosa with definite hyperparathyroidism in which parathyroidectomy was performed, a fall in plasma phosphatase corresponding with increasing calcification of the bones, as shown by x-ray, has been reported (15). With the resumption of

the normal rate of calcification in ricketic bone, a drop in serum phosphatase activity has also been observed (16).

In the absence of proof that nutritive failure precedes the fall in serum phosphatase activity of the rat subjected to magnesium deprivation, the decrease in serum phosphatase activity must be regarded as a primary result of magnesium deprivation. Furthermore, our data in conjunction with the observations of Kruse, Orent, and McCollum (5) with respect to the first 5 days of magnesium deprivation, indicate that the fall in serum phosphatase activity is closely related to the effects of magnesium deprivation on the osseous system.

#### SUMMARY

When rats, ranging from 25 to 48 days in age, were restricted to a magnesium-low diet, the subsequent decrease in serum magnesium was accompanied or closely followed by a marked fall in the serum phosphatase activity. The drop in the enzyme activity did not appear to be transitory, since it was observed in several rats of different ages at various intervals throughout an experimental period of 29 days.

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# THE SEXUAL VARIATION IN CARBOHYDRATE METABOLISM

## X. THE COMPARATIVE GLUCOSE TOLERANCE OF NORMAL RATS AND THOSE WITH FATTY LIVERS\*

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Carbohydrate metabolism shows certain qualitative differences in male and female rats. Not only are the fasting levels of liver glycogen consistently higher in male rats (1), but the amount of liver glycogen deposited in the liver of normal and adrenalectomized rats administered large amounts of glucose is also considerably more in the male (2). In the latter case the differences are not to be ascribed to a variation in the rate of absorption, since the higher rate of absorption seems to obtain in the female. It would seem probable that a higher level of blood sugar would be present in the female after the feeding of glucose if its removal as glycogen in the liver is slower.

Damage to the liver following chloroform or phosphorus poisoning has also been shown to result in elevated glucose tolerance curves in dogs (3). Moreover, Treadwell *et al.* (4) have recently found that there is a considerable reduction in glucose tolerance in rats having fatty livers produced by dietary means.

The present study was designed to follow not only the differences in response of male and female rats to a glucose tolerance test, but also the effect of fatty livers produced by nutritional means on such a response.

### EXPERIMENTAL

Male and female rats from our stock colony between 3 and 4 months old were used in the experiments. They were divided into four groups, two of which (Groups I and II) received our regular stock diet described elsewhere (5), while the other two groups received for 12 days the high fat, low protein diet employed earlier (6) except that 5 per cent of the cerelose was replaced with Cellu flour. Animals were fasted 24 hours before the glucose tolerance tests.

After removal of a sample of blood to determine the control level, 200 mg. of glucose per 100 gm. of body weight were given by stomach tube in 50

\* The data are from a thesis presented by Adelle Davis to the Graduate School of the University of Southern California in May, 1939, in partial fulfilment of the degree of Master of Science.

per cent solution. Samples of blood were taken at 30, 60, 90, and 120 minutes thereafter. The animals were then anesthetized with amytal, the liver removed, and the glycogen and lipid determined on aliquot samples of the liver.

Blood was collected from the tail in depressions on blocks of paraffin. Since preliminary tests showed that the blood sugar was elevated when the animal was moving or struggling, such movements were restricted by placing the rat in a thick glass tubing, large enough to permit comfortable entrance of the rat but no turning. Heavy wire netting was placed over one end and the opposite end was closed with a large cork through which a hole was cut for the animal's tail. Animals were placed in the tube 15 minutes before the first determination.

Blood sugar was determined by the Jegher and Myers (7) modification of the Folin micro blood sugar method, with the Klett-Summerson photo-electric colorimeter. Glycogen estimations were made by the technique of Good, Kramer, and Somogyi (8) with the Shaffer-Hartmann method. For the determination of liver lipids, the samples were dried to constant weight in a vacuum oven at 50°, after which they were extracted with diethyl ether on the Bailey-Walker extraction apparatus.

#### DISCUSSION

The results of the experiments are summarized in Table I. Table II includes the statistical evaluation of the data.

The blood sugar consistently reaches higher levels after the administration of glucose in female rats than in male animals. However, on the basis of the Fisher *t*, none of the averages of the postprandial samples of the rats on the stock diet shows a sex difference which is statistically significant; only the 90 minute sample obtained from rats with fatty livers gave a difference between the sexes which is statistically significant. When one makes the comparison on the basis of the increase in blood sugar over the fasting level, the variations are readily apparent with the groups receiving the high fat diet. While the actual rise in blood sugar over the fasting value in the males in Group III was 37, 31, 21, and 14 mg. respectively at the 30, 60, 90, and 120 minute periods, the corresponding increases in the blood glucose of female rats (Group IV) were found to be 58, 53, 62, and 35 mg. The first three of these averages are statistically greater than the values in the male.

Consistently higher blood sugar levels were noted after glucose in the rats having fatty livers than in the animals on the stock diet which had a normal liver lipid. These are significantly higher in all cases with the males but the actual extent of increase over the fasting level is almost identical in the two groups of male rats (Groups I and III). Thus, the successive

values in Group I are 35, 34, 24, and 10 mg. higher than the fasting level, while in Group III the corresponding rises are 37, 31, 21, and 14 mg. Also

TABLE I

*Blood Sugar of Rats Previously Fasted 24 Hours before and after Administration of 200 Mg. of Glucose per 100 Gm. of Body Weight*

Group No.	Previous diet	Sex	No. of tests	Average weight	Average age	Blood glucose, mg. per cent*					Liver glycogen	Liver lipid
						Fasting control	Time after glucose					
				30 min.	60 min.		90 min.	120 min.	per cent	per cent		
I	Stock	M.	10	gm. 219	days 113	92.6 ±6.1	127.9 ±6.3	126.1 ±6.6	116.1 ±5.9	103.0 ±6.1	0.74	4.30
II	"	F.	10	137	94	100.8 ±3.0	148.0 ±5.7	139.0 ±5.4	122.1 ±4.0	112.0 ±1.8	1.05	4.21
III	High fat	M.	12	185	116	119.0 ±4.2	156.1 ±3.5	150.0 ±3.2	140.5 ±4.1	132.7 ±6.9	0.97	21.7
IV	" "	F.	10	137	104	104.7 ±3.0	162.9 ±3.6	158.0 ±5.7	166.6 ±7.8	139.9 ±4.2	0.52	32.7

Increase in blood glucose over fasting level

I						35	34	24	10		
II						47	38	20	11		
III						37	31	21	14		
IV						58	53	62	35		

\* Including the standard error of the mean calculated as follows:  $\sqrt{\sum d^2/n}/\sqrt{n}$ , where  $d$  is the deviation and  $n$  is the number of determinations.

TABLE II

*Statistical Evaluation of Blood Glucose Values of Rats*

Comparison	Groups compared	Fisher <i>t</i> (9)					
		With <i>P</i> = 0.01	Blood samples				
			Fasting	30 min.	60 min.	90 min.	120 min.
Actual blood sugar	I:II	2.84	1.15	2.21	1.44	0.73	2.14
	III:IV	2.82	2.57	1.29	1.31	2.98	0.82
	I:III	2.82	3.45	3.88	3.30	3.36	3.04
Increase in blood sugar	II:IV	2.84	0.88	2.10	2.44	4.82	5.78
	III:IV	2.82		2.88	3.53	4.24	1.68
	II:IV	2.84		1.84	1.91	4.45	4.85

the blood sugar values are significantly greater in the 90 and 120 minute periods in the female rats which had fatty livers when compared with those animals on the stock diet with a normal fat content in the liver. The

elevations in Group II (stock diet) for successive periods were 47, 38, 20, and 11 mg. compared with values in Group IV (high fat diet) of 58, 53, 62, and 35 mg. respectively. The higher blood sugar values which obtain after the administration of glucose to rats with fatty livers (as compared with these values where the rats received the stock diet and had a normal lipid content) are suggestive of a mild diabetic type of tolerance curve. The greater severity of this phenomenon in the female is indicated not only by the higher actual level of blood sugar, but also by the greater elevation above the control level and the more prolonged period of hyperglycemia.

The greater elevation of blood sugar in the female is best explained by a slower glycogenesis, since there is no evidence of a marked variation in rate of absorption. After administration of doses of glucose which allow maximum absorption over as long a period as 8 hours, it was earlier demonstrated that there is a consistently higher level of liver glycogen in the male than in the female rat (2). Although this variation is also found in the present tests in the animals with fatty livers (in which the greatest sex differences in blood sugar were noted), the conditions were reversed in those previously on the stock diet. This discrepancy may be because equilibrium was not reached in the short period (2 hours) or because the doses were too small to bring about a maximum absorption over the whole time. However, not only have the sex differences in liver glycogen been found on fed animals but they have been noted on fasted rats by us (1) and others (10). The lowered carbohydrate reserve in the female also appears to be related to the greater susceptibility of women to fasting ketonuria (11) as well as the higher exogenous (12) and endogenous (6) ketonuria in the female rat.

These experiments add confirmation to those of Treadwell *et al.* (4) of the liver damage occasioned by the nutritional type of fatty livers. Johnston and Deuel<sup>1</sup> have also noted that the ketolytic action of substances may be lowered under conditions in which fatty livers occur. These authors have shown that under such conditions, sorbitol which normally brings about a greater glycogenesis in the liver than glucose, becomes markedly inferior as a glycogenic agent.

#### SUMMARY

In rats having fatty livers the extent of increase in blood glucose was greater in female than in male animals.

The blood glucose levels are significantly higher in the rats of both sexes having fatty livers. In the males, the extent of increase over the fasting level is almost identical in the rats with and without fatty livers. However, a significantly greater rise in blood sugar occurs after glucose in the

<sup>1</sup> Johnston, C. H., and Deuel, H. J., Jr., unpublished observations.

female rats with fatty livers than in those possessing a normal fat quota in the liver.

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# A PROCEDURE FOR THE DETERMINATION OF ASCORBIC ACID BASED UPON THE USE OF A STANDARDIZED SOLUTION OF 2,6-DICHLOROPHENOL INDOPHENOL IN XYLENE

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The outstanding property of ascorbic acid is its strong reducing power, and most methods for its determination are based on this property. Tillmans, Hirsch, and Hirsch (1) introduced the use of 2,6-dichlorophenol indophenol as oxidizing agent for the titration of ascorbic acid. This method has been extended and developed by Bessey and King (2), Farmer and Abt (3), and others. In these procedures a standardized solution of the oxidized dye is titrated into an acid solution containing an unknown amount of ascorbic acid. Determination in this way is limited to clear, uncolored solutions. There is the possibility of error due to slow reduction of the dye by substances other than ascorbic acid. If the acidity of the solution titrated is too high, the dye fades owing to the acidity. The color end-point of the titration is not sharp and requires considerable experience for accurate work.

Mindlin and Butler (4) have developed a procedure for the determination of ascorbic acid in plasma based upon photoelectric measurement of excess dye remaining in metaphosphoric acid filtrates after reaction between ascorbic acid and 2,6-dichlorophenol indophenol. These workers buffered their filtrates to pH 4.10 with sodium acetate and permitted reaction with the dye for only 30 seconds before taking readings. This procedure reduces errors due to excess acidity and slowly reducing substances. Bessey (5) has modified the method as used by Mindlin and Butler (4) by eliminating the necessity of clear and colorless filtrates.

The discovery that oxidized 2,6-dichlorophenol indophenol can be quantitatively extracted from acid solution with xylene has resulted in the development of rapid methods for the determination of ascorbic acid by Bukatsch (6) and Stotz (7). In these methods the dye is allowed to remain in contact with the ascorbic acid solution for a short time (15 to 30 seconds), extracted into xylene by shaking and centrifugation, and the unreduced dye estimated by colorimetric reading. The oxidized indophenol in xylene is not affected by the acid solution or slowly reducing substances.

\* Taken from the thesis of Doris M. Higher presented in partial fulfillment of the requirements for the degree of Master of Science. Aided by a grant from the General Research Council of the Oregon State System of Higher Education.

The purpose of this report is to describe the development of a simple general method for the determination of ascorbic acid utilizing the solubility and stability of oxidized 2,6-dichlorophenol indophenol in xylene. While Bukatsch (6) and Stotz (7) added the dye to the solution to be tested, followed by extraction of unreduced dye with xylene and colorimetric reading, in the method reported here a standardized solution of dye in xylene which is shaken directly with the solution to be analyzed is used, followed by photoelectric measurement of unreduced dye remaining in the xylene layer.

#### EXPERIMENTAL

*Apparatus*—Klett-Summerson photoelectric colorimeter (8) with filter No. 540.

*Reagents*—

1. 0.03 N HCl.
2. Xylene, c.p.
3. Saturated solution of rosin in kerosene. An excess of powdered rosin is allowed to stand in kerosene for several days. The solution is then filtered.

4. 2,6-Dichlorophenol indophenol in xylene. 0.1 gm. of 2,6-dichlorophenol indophenol is extracted with two 25 cc. portions of boiling water, filtered, and diluted to 200 cc. When cool the solution is acidified with 0.03 N HCl until red. 200 cc. of xylene are then added, and the mixture is shaken. The xylene-dye solution is washed by shaking with several 200 cc. portions of 0.03 N HCl. Careful washing prevents fading of the dye by the acid present in the solution to be analyzed. The xylene layer is separated and placed in a clean dry flask, and dried by shaking with anhydrous sodium sulfate, followed by filtration. A portion of the concentrated xylene-dye solution is diluted to a definite photoelectric reading. A value of 150 was found satisfactory for our work. If the dilution is carried too far, the proper amount of more concentrated solution is added. Some concentrated solution should be reserved for this purpose. Xylene-dye solutions prepared in this way are quite stable if protected from prolonged exposure to light.

After each analysis the xylene-dye and aqueous solutions are poured into a bottle. When considerable volume has accumulated, the mixture is filtered through a cotton plug and treated as follows: The xylene-dye solution is separated from the aqueous layer, washed several times with 0.03 N HCl, shaken with anhydrous sodium sulfate, filtered, and brought to standard reading by the addition of concentrated dye in xylene. Mixtures containing stannous chloride could not be adequately purified by this procedure. Dye solutions recovered in such cases were always faded by shaking with 0.03 N HCl.

*Procedure 1. Solutions Not Containing Protein*—1 to 3 cc. of solution containing not more than 0.06 mg. of ascorbic acid is pipetted into a 75 cc.  $8 \times 1$  inch test-tube and diluted to 15 cc. with 0.03 N HCl. 10 cc. of the standardized xylene-dye solution are added. The test-tube is fitted with a rubber stopper and the mixture shaken for 15 seconds (50 to 60 excursions of a  $90^\circ$  arm sweep), poured into a 50 cc. centrifuge tube, and centrifuged for 5 minutes at 1000 R.P.M. 5 cc. or more of the xylene-dye solution are then poured into a colorimeter tube and read after the photoelectric colorimeter has been adjusted to zero for xylene. The above procedure is applicable to the determination of ascorbic acid in deeply colored fruit juices

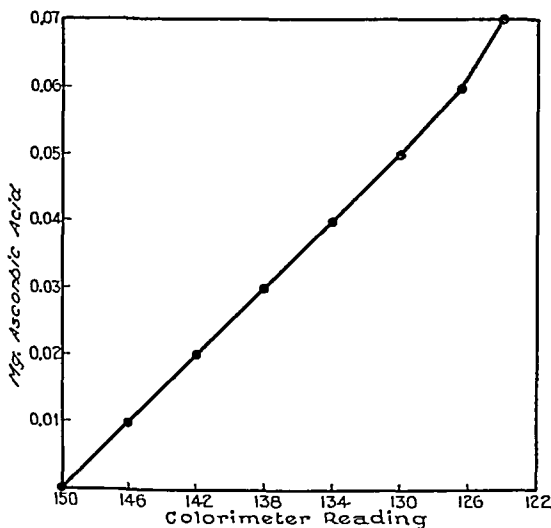


FIG. 1. Concentration curve of ascorbic acid

(blackberry, huckleberry), citrus fruit juices, blood filtrates, urine, and other aqueous solutions not containing protein.

Fig. 1 gives colorimeter readings plotted against known ascorbic acid concentrations as determined by Procedure 1.

*Procedure 2. Determination of Ascorbic Acid in Serum and Plasma*—The procedure is exactly as given in Procedure 1 with the exception that 1 cc. of the rosin solution is added immediately after addition of the 0.03 N HCl. Owing to the low concentration of ascorbic acid in blood serum and plasma, it is advisable to use 2 to 3 cc.

It was found that, when 1 cc. of rosin in kerosene was added to 10 cc. of xylene-dye solution reading 168, the dye solution was diluted to a

reading of 150. Accordingly, by using a dye solution reading 168, it was possible to compensate for dilution by the rosin solution and to use the curve obtained by Procedure 1. Since rosin solutions may vary, the dilution effect should be determined in each case and the reading of the dye solution adjusted accordingly.

Previous work referred to showed that there are reducing substances other than ascorbic acid present in many biological solutions but that these substances generally reduce more slowly than does ascorbic acid. Because of this situation, less error, due to these non-ascorbic acid substances, is encountered if the dye is in contact with the solution analyzed for only a short time. In results reported in this paper the reaction time was 15 seconds.

Aqueous solutions containing 4 mg. per cent of reduced glutathione, cysteine hydrochloride, tannic acid, pyrogallol, and high dilutions of strong acids do not fade the xylene-dye when shaken with it for 15 seconds.

TABLE I  
*Concentration of Ascorbic Acid in Various Juices*

Juice sample (1 cc.)	Dilution of sample	Colorimeter reading	Ascorbic acid
			mg. per cent
Orange.....	1:15	137	49
Lemon.....	1:15	135	56
Grapefruit.....	1:15	140	37
Blackberry (canned)....	None	138	3
Huckleberry " .....	"	126	6

Preliminary work with fruit juices, including lemon, orange, grapefruit, blackberry, and huckleberry juice, showed that they cause fading of color when shaken with a solution of xylene-dye. Since these juices contain pigments, it was necessary to determine the solubility of these in xylene. When tested by the method, 10 cc. of the solvent being substituted for the xylene-dye, all gave zero readings, showing that the pigments do not interfere directly in the estimation of ascorbic acid by this method. A series of values for various fruit juices obtained by Procedure 1 is given in Table I.

The method as used for the assay of pure ascorbic acid was found applicable to the determination of ascorbic acid in 2 per cent metaphosphoric acid filtrates of blood plasma and blood serum. However, since the concentration of ascorbic acid in these filtrates is very low, 0.4 to 2.5 mg. per cent, it is necessary to use an amount of filtrate equivalent to at least 1 cc. of the original fluid tested, followed by dilution to 15 cc. with 0.03 N HCl. 2 per cent metaphosphoric acid does not fade the dye.

Direct measurement of ascorbic acid in blood plasma and serum was attempted with metaphosphoric acid as the acidifying agent. It was found

in each case that precipitation of the blood proteins by the acid resulted in the formation of an emulsion during shaking which prevented separation of the xylene-dye solution. Attempts to prevent the formation of these stable emulsions by the addition of various substances were unsuccessful. It was found that when 0.03 N HCl is substituted for metaphosphoric acid

TABLE II  
*Concentration of Ascorbic Acid in Samples of Plasma and Serum*

Sample analyzed		Condition of sample	Colorimeter reading	Ascorbic acid
	cc.			mg. per cent
Serum	1	Old	150	0.00
	1	2 days in ice box	148	0.50
	1	Fresh	144	1.50
	2	"	145	0.67
Plasma	1	"	144	1.50
	1	"	145	1.26
	2	"	139	1.37
	3	"	140	0.87
	3	"	137	1.08

TABLE III  
*Comparison of Plasma Ascorbic Acid Values Determined by Xylene-Indophenol Method and by Method of Farmer and Abt (3)*

Sample No.	Xylene-dye method			Farmer and Abt method
	Sample analyzed	Colorimeter reading	Ascorbic acid	Ascorbic acid
	cc.		mg. per cent	mg. per cent
1	1	144	1.50	1.59
2	1	146	1.00	0.91
3	1	145	1.25	1.32
4	1	147	0.75	0.72
5	1	146	0.87	0.82
6	2	142	1.00	1.00
6	2*	149	1.00	
7	2	144	0.75	0.74
8	2	143	0.87	0.88

\* Metaphosphoric acid filtrate of plasma.

no precipitation of proteins occurs but a stable emulsion is formed during shaking, which prevents separation of the xylene-dye solution. The stability of this emulsion was investigated and it was found that, if a solution of rosin in kerosene is added to the sample in 0.03 N HCl before addition of the xylene-dye solution, the latter may be readily separated.

The stability of ascorbic acid in 0.03  $\times$  HCl was investigated. No destruction was observed over a period of 1 hour at room temperature. Shaking the indophenol-xylene solution 15 seconds with 0.03  $\times$  HCl causes no fading of the dye. These findings led to the use of 0.03  $\times$  HCl in the determination of ascorbic acid in all solutions which are not required to stand more than 1 hour before assay.

TABLE IV  
*Concentration of Ascorbic Acid in Samples of Urine*

Sample No.	Condition of sample	Colorimeter reading	Ascorbic acid	Ascorbic acid after 24 hrs. with Sendroy's reagent
			mg. per cent	mg. per cent
1	Fresh	138	3.00	
2	"	143	1.75	
3	"	147	0.75	0.75
4	"	138	3.00	3.00
5	"	138	3.00	2.75
6	"	135	3.75	3.75
7	"	131	4.25	4.25
8	24 hrs.	150	0.00	
9	Fresh	141	2.25	
	24 hrs.	143	1.75	

TABLE V  
*Recovery of Ascorbic Acid Added to Various Fluids*

Fluid	Original ascorbic acid to which 1 mg. per cent was added	Ascorbic acid found
	mg. per cent	mg. per cent
Urine.....	2.5	3.5
" .....	2.0	3.0
Orange juice.....	45.0	46.0
Blood serum.....	1.25	2.25

Plasma from oxalated blood which had been agitated as little as possible and immediately chilled, and serum taken as soon as clotting permitted, followed by chilling, were tested according to Procedure 2. The results are given in Table II.

Table III gives a comparison of ascorbic acid values in plasma as determined by the xylene-dye method and the method of Farmer and Abt (3). The special micro burette of Farmer and Abt was not available and an ordinary 5 cc. micro burette was used. The results show good agreement when 2 cc. of plasma were used for determination by the xylene-dye method.

Many preservatives were used in an attempt to prevent destruction of ascorbic acid in urine. Specimens containing 5 per cent  $\text{HPO}_3$ , kept at room temperature, and those containing 2 per cent  $\text{HPO}_3$ , kept in the ice box, showed no destruction of ascorbic acid in 24 hours. Since the cost of  $\text{HPO}_3$  is high, its use for a large number of samples is rather expensive.

24 hour specimens of urine containing a modification of Sendroy's reagent showed very little or no destruction of ascorbic acid when kept at room temperature. Sendroy's reagent as modified and used for the preservation of ascorbic acid in urine is as follows: In each container used for a 24 hour collection there are placed 75 cc. of 5 N sulfuric acid and 5 cc. of 8-hydroxyquinoline solution (1.45 gm. of 8-hydroxyquinoline in 100 cc. of alcohol).

Table IV summarizes results obtained on various urine samples. Preservative was used only as indicated. 1 cc. samples were used for analysis.

TABLE VI  
*Effect of Ascorbic Acid Oxidase on Xylene-Dye Readings*

Fluid	Sample analyzed	Initial colorimeter reading	Ascorbic acid	Colorimeter reading after oxidase	Ascorbic acid
	cc.		mg. per cent		mg. per cent
Urine.....	1	145	1.25	150	0.00
" .....	1	146	1.00	150	0.00
" .....	1	138	3.00	150	0.00
Serum.....	2	144	0.75	150	0.00
" .....	2	143	0.87	148	0.25
" .....	2	139	1.37	150	0.00

Ascorbic acid was added to various biological solutions of previously determined concentration. The recovery is shown in Table V.

Ascorbic acid oxidase, as prepared and used by Sharp, Hand, and Guthrie (9), was used to test the specificity of the method (Table VI). 0.1 cc. of the enzyme solutions used was capable of oxidizing completely 0.05 mg. of ascorbic acid in a volume of 5 cc. when permitted to stand at room temperature for 30 minutes.

The method has been satisfactorily applied to the determination of ascorbic acid in metaphosphoric acid filtrates of ground animal tissues.

#### SUMMARY

A simple method for the estimation of ascorbic acid in biological fluids based upon use of a standardized xylene solution of 2,6-dichlorophenol indophenol is described.

The method has been found applicable to the determination of ascorbic



acid in various fruit juices including highly colored juices, urine, and blood plasma or serum. The analysis of plasma and serum ascorbic acid may be carried out directly upon the material without preparation of a filtrate.

The method may be applied to metaphosphoric acid filtrates of tissues.

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# ELECTROPHORETIC PATTERNS, COLLOID OSMOTIC PRESSURE, AND VISCOSITY OF SERUM DENATURED BY ULTRAVIOLET RADIATION

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It has long been known that ultraviolet radiation is one of the numerous agents which may denature proteins, and, as with other forms of denaturation, comparatively little is known of the precise molecular changes which are involved. Various reports describe as a consequence of irradiation an increase in viscosity of globulin (1), the appearance of sulfhydryl groups in egg albumin (2), a decrease in trichloroacetic acid-precipitable nitrogen in egg albumin, and an increase in dialyzable nitrogen (3, 4). Svedberg and Brohult (5) showed that irradiation of hemocyanin resulted in the splitting of this protein into half molecules; irradiation of hemoglobin, however, resulted in an inhomogeneous mixture of molecules both larger and smaller than normal. Sanigar *et al.* (6) found that irradiation caused aggregation and inhomogenization of human serum albumin. The irradiation of aqueous solutions of thymonucleic acid (7) resulted in a marked decrease in the properties of structural viscosity and streaming birefringence, which was interpreted as being due to a splitting of the thymonucleate into smaller, less asymmetric particles.

In the present study we have investigated by the use of several criteria the effect of irradiation on serum and on certain protein fractions of the latter. The methods include viscosity determinations at various external pressures, electrophoretic analyses, and determinations of colloid osmotic pressure.

## Procedure

The experiments were performed with undiluted horse serum, and with total protein, albumin, and globulin fractions obtained from human serum. The samples were divided in half, one portion being irradiated at 21–25° with occasional stirring in a stoppered quartz tube, the other portion being irradiated simultaneously in a stoppered Pyrex tube. The radiation consisted of 80 per cent at 2537 Å., the residual 20 per cent being distributed over the spectrum from 2400 to 10,000 Å. For details of the experimental technique and energy values see Hollaender *et al.* (7).

The viscosity was determined in a Bingham and Jackson viscometer

at  $30^{\circ} \pm 0.01^{\circ}$  at various external pressures. The results are expressed in terms of relative viscosity, with water as the standard of reference. The electrophoretic patterns were obtained in the Longsworth (8) modification of the apparatus of Tiselius. Except where otherwise stated, electrophoresis took place in a buffer at pH 7.7 of 0.01 M phosphate plus 0.1 N NaCl.

The colloid osmotic pressure was determined at room temperature by the method of Hepp (9, 10), a collodion membrane being used. In this method the principle of connecting to the fluid in a fine capillary the thin layer of ultrafiltrate beneath a large membrane is employed. The pressure in the capillary is counterbalanced by a varying hydrostatic pressure until equilibrium is attained. Equilibrium is recognized by the absence of microscopically visible movement of the capillary meniscus.

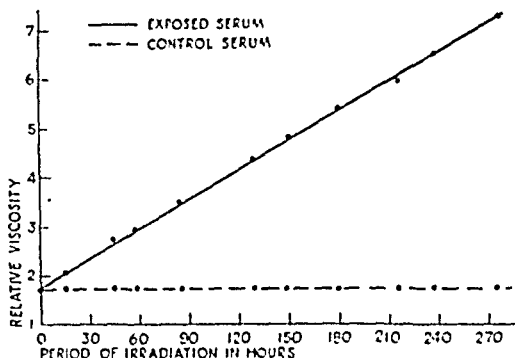


FIG. 1. The relative viscosity of horse serum (5.5 per cent) as a result of ultra-violet radiation.

Because of the large filtering surface this method is quite rapid, giving the value of the colloid osmotic pressure within 15 to 20 minutes to an accuracy of 1 per cent. The value remains constant usually for as long as 12 hours. Successive samples may replace each other on the same membrane, since the ultrafiltrate occupies only the small volume of a layer of filter paper beneath the membrane and hence rapidly readjusts itself to the new sample.

### Results

*Viscosity*—The progressive nature of the increase in viscosity with time of irradiation is shown in Fig. 1. The sample of horse serum was irradiated for about 280 hours. At intervals during this period the viscosity of both test and control samples was measured at an external pressure of 16.0 cm. of water. The viscosity of the control serum (irradiated in Pyrex) did

not change appreciably during the irradiation period. On the other hand, there was a linear increase in the viscosity of the irradiated serum until about 11 days of irradiation, when the viscosity had increased almost 5 times over that of the control. The latter remained clear throughout. The exposed serum became progressively more opalescent and then turbid during the irradiation period until about the 12th day, when precipitation began. No appreciable changes occurred in the content of inorganic phosphate and ammonia in either test or control samples. The pH decreased from 7.3 to 7.0 in the former sample.

At the end of the irradiation period, the viscosity of both portions of serum was determined at various external pressures (11). No change in

TABLE I  
*Effect of Irradiation on Colloid Osmotic Pressure*

Sample	Protein concentration	Period of irradiation	Relative viscosity*	Colloid osmotic pressure
	per cent	hrs.		mm. H <sub>2</sub> O
Horse serum (control).....	5.5		1.67	258-284
" " (test).....	5.5	24	2.25	207
" " ".....	5.5	120	4.10	140
" " ".....	5.5	72	3.20	158
" " ".....	5.5	144	4.68	155
" " ".....	5.5	168	7.63	120
Human serum† (control).....	6.48		1.64	331
" " † (test).....	6.48	40	3.46	110
" " albumin (control).....	6.24		1.45	398
" " " (test).....	6.24	40	3.09	121
" " globulin (control).....	6.96		2.27	189
" " " (test).....	6.96	40	3.82	134

\* At 16.0 cm. of H<sub>2</sub>O external pressure.

† Human serum total protein in saline-phosphate buffer.

viscosity with pressure was evident in the control serum. On the other hand, there was a small but definite drop in the viscosity from 7.84 at 4.4 cm. of H<sub>2</sub>O to 7.16 at 20 cm. of H<sub>2</sub>O in the case of the irradiated serum; i.e., the phenomenon of structural viscosity. The latter property suggests an over-all increase in the asymmetry of the protein molecules of the irradiated serum.

*Colloid Osmotic Pressure*—The colloid osmotic pressure of serum and of the serum proteins markedly decreased with irradiation. The results of representative experiments are given in Table I. Irradiation of horse serum for 3 to 7 days approximately halved the osmotic pressure.

The values given for the colloid osmotic pressure of the irradiated sam-

ples, which are those recorded after  $\frac{1}{2}$  hour of equilibration, are only approximate because of the anomalous behavior of this material. Whereas the osmotic pressure of normal serum deviates by only 1 to 2 mm. of  $H_2O$  for hours after equilibration, the irradiated serum showed a progressive decrease of pressure with time. Thus a sample which measured 120 mm. after  $\frac{1}{2}$  hour of equilibration in the osmometer, had decreased to 110 mm. by 1 hour and 90 mm. by 12 hours. This may be explained by a progressive aggregation of the molecules in the test samples, by possible thixotropic behavior of the asymmetric denatured molecules, or by the presence of molecules small enough to escape slowly through the membrane. We are inclined to the latter view because of the fact that when irradiated serum was replaced on the membrane by normal serum of known osmotic pressure the pressure observed after the usual period of equilibration was lower than expected, and returned to the normal value only after several hours. This suggests that the ultrafiltrate from the denatured protein contained particles which slowly distributed themselves across the membrane when a new solution was introduced, and which lowered the observed pressure until that distribution was complete.

Reduction of osmotic pressure and increase in viscosity (at 16 cm. of  $H_2O$  pressure) were observed also in the case of the human serum albumin and globulin fractions. The globulin fraction, however, showed a smaller relative change in osmotic pressure and in viscosity for the same period of irradiation than did the albumin or total serum protein solutions. This may be due to diminished penetration of light into the globulin solution, which was somewhat opalescent at the start of the experiment, and became more so during irradiation. The other solutions were clear but became opalescent during irradiation.

The marked reduction in the colloid osmotic pressure of the samples following irradiation indicates that the average molecular weight of the proteins in these samples is considerably greater than in samples of normal sera, this increase most probably being due to molecular aggregation.

*Electrophoretic Patterns*—Electrophoresis of the irradiated sera showed a progressive increase in homogeneity of the material, with formation of a new peak in the middle of the existing components (Fig. 2). This new peak (labeled *D* for denatured) appears to be formed from the other components in the proportions in which they are present. Evidence for this is furnished by the fact that the *D* peak of 1 day-irradiated serum has the same mobility as that of 5 day-irradiated serum, although in the latter case a much larger proportion of the protein had been denatured. Proof of the identity of the mobility was obtained in an electrophoretic analysis of a mixture of two samples which showed only one *D* component.

The *D* peak is very narrow and steep. It is even steeper than an albumin

peak of the same total area. This striking sharpness may be partly due to a decreased diffusion constant which would be expected from the increased mean molecular weight and molecular asymmetry shown by the colloid osmotic pressure and structural viscosity. To insure that the observed homogeneity was not a coincidence due to crossing of mobility curves of more than one component at the pH studied, one sample of serum was also studied in phosphate buffer at pH 6.7 and in barbiturate at pH 8.5. The results were essentially the same as those found at pH 7.7.

Mobilities were not measured in absolute units in this study, because compensatory hydrostatic shifting of the boundaries was necessary to obtain maximal separation of the components. In addition, since the mobility of a protein depends somewhat on the concentration of the other components around it, a truer estimate of the mobility of an unknown substance may be obtained by comparing it with substances of known mobility, *i.e.*, viewing it against normal serum as a frame of reference. Such an experiment is seen in Fig. 2, in which a mixture of equal volumes of normal and of 3 day-irradiated serum was analyzed. It is seen that the *D* peak lies at about the position of  $\beta$ -globulin—the position which would be expected for a weighted mean of the mobilities of all the components present. The smaller components of the serum are obliterated by the base of the *D* component.

To test the tentative hypothesis that the denatured protein represents electrophoretically a "fusion" of the material present, a sample of human serum was separated by ammonium sulfate into the conventional albumin and globulin fractions, dialyzed until salt-free, and finally dialyzed against 0.15 *N* NaCl buffered at pH 7.4 by 0.01 *M* phosphate. The samples were simultaneously irradiated for 40 hours. The globulin fraction underwent less change than the albumin (Table I). On electrophoretic analysis the albumin fraction, which was admixed with a small per cent of the slower globulins, ended after irradiation as essentially a single component moving slightly more slowly than the original albumin (Fig. 3). The globulin fraction, which consisted predominantly of slow components, ended similarly as a denatured component of approximately the mean mobility of the original components; *i.e.*, slightly slower than that of  $\beta$ -globulin. In all these cases, the relative homogeneity of the denatured component was established by electrophoresis of the irradiated material, and the mobility by electrophoresis of a mixture of this with normal serum.

The extent of denaturation produced in the human serum protein and its two fractions by only 40 hours of irradiation, judged by any of the three criteria here used, was comparable to that produced by several days of irradiation of native horse serum. The human serum proteins, however, were in saline-phosphate buffer rather than in their natural environment.

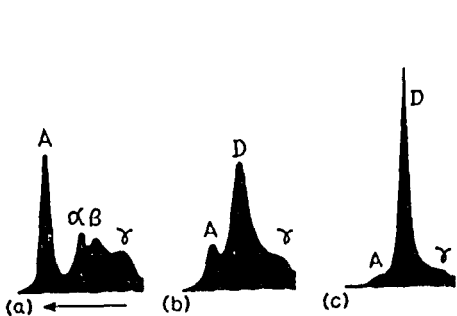


FIG. 2

FIG. 2. Diagrams of ascending boundaries, which were very symmetrical with descending boundaries. Phosphate buffer, pH 7.7. (a) Control horse serum. (b) Irradiated 1 day. (c) Irradiated 3 days. (d) Irradiated 5 days. Note the progressive increase, with irradiation, of the size and sharpness of the *D* boundary. (e) Mixed equal amounts of normal serum (a) and 3 day-irradiated serum (c). Note the close resemblance to 1 day-irradiated serum (b). (f) Mixed equal amounts of serum irradiated 1 day (b) and 5 days (d). Note that the mobility of the *D* component remains unaltered with further irradiation, for only one sharp peak is present.

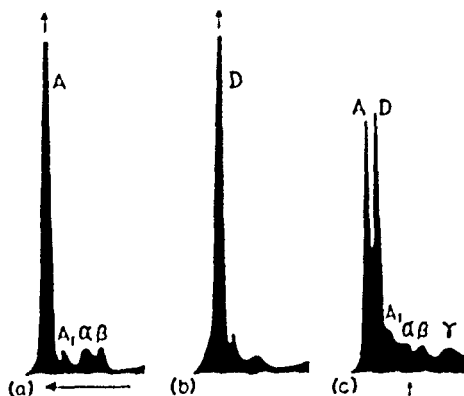


FIG. 3

FIG. 3. (a) Human serum "albumin." Note the admixture of true albumin with some  $\alpha$  and  $\beta$ .  $A_1$  is the small portion of albumin which migrates more slowly than the main portion in barbital buffer of pH 8.5, in which all human samples were run. (b) Irradiated human serum albumin. (c) Mixed equal amounts of irradiated serum albumin and native human serum. Note that the *D* peak migrates slightly more slowly than the *A* peak. (d) Human serum "globulin." Irradiated globulin was too turbid to photograph well. (e) Mixed equal amounts of irradiated serum globulin and native human serum. Note that the *D* peak of globulin migrates much more slowly than that of albumin. (f) Heat-denatured horse serum.

In another experiment on human serum prepared from citrated plasma by removal of the fibrin clot produced by the addition of  $\text{CaCl}_2$  to a concentration of 0.05 M, complete coagulation was produced by only 4 hours of

irradiation. It appears that the denaturation produced by ultraviolet radiation may be affected by the ions present in the solution.

To compare the effects of irradiation with those of heating, a sample of horse serum was heated at 60–65° for 8 hours, or until it had attained approximately the same degree of opalescence as the 5 day-irradiated material. The increased viscosity and electrophoretic homogeneity were very similar to those found after irradiation, but the drop in colloid osmotic pressure was of a lower order of magnitude.

#### DISCUSSION

The colloid osmotic pressure is a true measure of the mean molecular weight only when corrected for the non-ideal behavior of protein solutions and for Donnan equilibrium. The former correction is attainable by extrapolating to infinite dilution the values obtained by a series of dilutions, since protein solutions with increasing concentration exert a greater pressure per unit concentration. This correction accounts for roughly 40 per cent of the colloid osmotic pressure of serum; we assume that denaturation does not alter the order of magnitude of this correction.

The Donnan equilibrium similarly accounts for about 20 per cent of the colloid osmotic pressure of serum; it may be eliminated by measurement at the isoelectric point, which cannot be done with denatured proteins because of their insolubility. From the fact, however, that the mean electrophoretic mobility of the denatured protein is about the same as that of the native protein, we know that there has been no significant change in the base bound and hence in the Donnan effect. In terms of colloid osmotic pressure, this constant Donnan correction becomes a larger proportion of the lower values obtained with the irradiated material. Hence, when the colloid osmotic pressure is halved following irradiation, the mean molecular weight is at least doubled.

The remarkable electrophoretic homogeneity of the denatured serum suggests a corresponding homogeneity in composition (though not necessarily in particle size). There are several ways in which denaturation might alter electrophoretic mobility. First there is the alteration in the state of aggregation, as demonstrated by the colloid osmotic pressure. But there is no theoretical reason to expect particle size to affect mobility, provided the charge density and hence the  $\zeta$  potential remain constant. Denaturation may lower mobility by increasing the molecular asymmetry; Abramson, Gorin, and Moyer (12) have presented calculations to indicate the effect of particle shape on mobility. Thirdly, it may alter mobility by changing the number of ionizable groups and the dissociation constants of such groups. Finally, denaturation might affect mobility by altering the actual composition of the particle in terms of amino acids. From the fact that



the irradiation of our preparation of "albumin" produced only the slight shift in mobility of the major component (true albumin), which would be expected from homogenization with the contaminating slower components, we assume that denaturation under these conditions has very little effect on the mobility of an electrophoretically pure substance. The changes observed would then be largely due to the chemically homogenizing process, rather than to changes within the molecule.

This process may take place in two ways. The first would be aggregation of the molecules in the proportions in which they are present, so that eventually all the particles present would be very large ones, with the same charge density. To produce the degree of electrophoretic homogeneity observed, such particles would have to be very large and would therefore contribute very little to the colloid osmotic pressure. The alternative mechanism would involve simultaneous splitting and recombination of the molecules, achieving electrical homogeneity without the exclusive formation of huge molecules.

It is likely that splitting takes place, for the serum had retained half its colloid osmotic pressure when it had become 90 per cent homogeneous. There is reason from other sources, cited in the introduction (3-6), to believe that ultraviolet irradiation may involve fission of certain linkages. Our interpretation of the data at hand, then, is that ultraviolet denaturation involves first an unfolding and then a splitting and an aggregation of the molecules, producing molecules of similar charge distribution but widely varying particle size. The lowered colloid osmotic pressure would indicate a predominance of large aggregates.

In this connection it is of interest to consider reports by other investigators of changes produced in serum and in protein fractions thereof by other denaturing agents. Van der Scheer, Wyckoff, and Clarke (13) found that the heating of serum at 65° for a relatively short period of time resulted in the formation of a single colloidal component at the expense of the globulin and albumin components of the serum. We have confirmed this finding, and have also demonstrated the similar phenomenon produced by ultraviolet radiation. The work of Neurath and coworkers (14) on the denaturation of the serum proteins by urea and by guanidine hydrochloride has shown that these reagents produce a marked increase in the relative viscosity and a decrease in the diffusion constant. At 2 M guanidine hydrochloride an aggregation of the pseudoglobulin molecules occurs, the mean molecular weight being about twice that of the native protein. At 3 M concentration of the denaturant, the pseudoglobulin molecules split into halves as they unfold. It may be stated in general that the effect of various denaturing agents on the serum proteins is similar, but the magnitude of the results obtained will vary with the intensity and nature of the agent employed.

## SUMMARY

1. Ultraviolet irradiation of horse and of human serum and of certain protein fractions of the latter results in a marked increase in relative viscosity and decrease in colloid osmotic pressure. The electrophoretic pattern becomes homogeneous, with approximately the mean mobility of the components initially present.

2. In conjunction with evidence from other sources, it is concluded that the major effect of irradiation on serum proceeds through unfolding and splitting of the protein molecules with subsequent aggregation.

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# THE RÔLE OF POTASSIUM IN MUSCLE PHOSPHORYLATIONS\*

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A wide number of observations, reviewed by Fenn (1) recently, have indicated the importance of potassium in muscle physiology. Although numerous physical and chemical explanations have been advanced, the mechanism of potassium action has remained obscure. A relationship of potassium to carbohydrate metabolism has been indicated by several investigations. The existence of a cycle in which potassium accompanies carbohydrate from muscle to liver and other viscera and back again has been suggested (2). Potassium has been found to increase the anaerobic fermentation of yeast and tumor tissue (3, 4). Aerobic glycolysis by brain tissue is increased considerably by addition of an excess of potassium (5, 6). Pulver and Verzár (7) have observed a close relation between potassium and glycogen formation in yeast. These effects of potassium on carbohydrate metabolism have been explained by a possible effect on glycogen synthesis or breakdown (7, 8).

Recently experiments were undertaken in this laboratory to study the phosphorylation of creatine by muscle tissue. Needham and van Heyning-en (9), and Lehmann (10) have demonstrated phosphorylation of creatine from 3-phosphoglycerate and 2-phosphopyruvate by the use of old, well dialyzed, muscle extracts, additions of magnesium and adenylic acid being essential for phosphorylation. Belitzer (11) has obtained phosphorylation of creatine with carefully prepared, fresh, minced muscle tissue, with lactate and malate additions.

During the course of our studies it was found that potassium was essential for the transfer of phosphate from 3-phosphoglycerate to creatine by muscle extracts. Experiments were thus conducted to study in more detail the relation of potassium to the phosphate transfers involved in the phosphorylation of creatine.

## *Methods and Materials*

Muscle tissue preparations were prepared from rat muscle removed immediately after the animal was killed by a blow on the head, or while it was under ether anesthesia, a section from the mid-portion of the gastrocnemius

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being used. The tissue was rapidly weighed, and homogenized either with the phosphate buffer used or with distilled water by grinding with a pestle in a mortar with a small amount of washed sand. Minces, when used, were prepared by careful mincing with small scissors and suspending in phosphate buffer. The phosphate buffers used, unless otherwise indicated, were prepared by using appropriate amounts of  $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$ .

Phosphocreatine was determined by the calcium precipitation method of Fiske and Subbarow (12) after deproteinization with 10 per cent trichloroacetic acid and removal of the precipitate by centrifuging rapidly at  $0^\circ$ .

The adenosine triphosphate was prepared by a modification of Lohmann's procedure (13) as used by Cori.<sup>1</sup> The 3-phosphoglyceric acid was prepared from fluoride-treated yeast by the method of Neuberg and Kobel (14).<sup>2</sup> Phosphocreatine was synthesized by the method of Kiessling (15). 2-Phosphopyruvic acid was prepared by the method of Zeile and Fawcett (16).

The adenosine triphosphate, 3-phosphoglycerate, and 2-phosphopyruvate, obtained as barium salts, were freed of barium by dissolving in a minimum amount of  $\text{HCl}$ , adding slightly more  $\text{Na}_2\text{SO}_4$  than was necessary for complete barium precipitation, centrifuging off the precipitate, and neutralizing with  $\text{NaOH}$  to approximately pH 7.4 (brom-thymol blue). All substrates and other additions were neutralized before use.

The boiled muscle juice was prepared by dropping the muscle freshly removed from the front and hind legs of a rat into about 50 cc. of boiling water, boiling for  $1\frac{1}{2}$  to 2 hours, and straining through several layers of cheese-cloth. After most of the trials on the stimulation of phosphorylation by muscle juice had been completed, it was noted that muscle juice additions caused some interference in the phosphocreatine determination. This effect was tested on solutions of phosphocreatine, and it was found that additions of 0.3 cc. of muscle juice per cc. of solution decreased the amount of phosphocreatine measured by roughly one-fourth. Thus the observed stimulation of phosphorylation was probably somewhat greater than the results given indicate. In further studies it was possible to replace the muscle juice by potassium, and thus this interference was not further investigated.

### Results

Studies on creatine phosphorylation were first made to ascertain whether phosphorylation could be obtained with fresh muscle extracts under conditions similar to those used by Needham and van Heyningen (9), Lehmann (10), and Belitzer (11), with either homogenized or minced preparations.

<sup>1</sup> Cori, G. T., personal communication.

<sup>2</sup> Kindly supplied by Dr. P. L. Pavcek.

In trials with muscle homogenates and lactate and malate additions, no phosphocreatine synthesis was obtained with conditions as shown in Table I. Additions of boiled muscle juice, for other possible activators, did not stimulate synthesis by homogenized tissue but made the synthesis possible by minced tissue. With 3-phosphoglycerate additions, in the absence of boiled muscle juice, synthesis could be obtained with minced but not with homogenized tissue. Synthesis of phosphocreatine by minces under these

TABLE I

*Effect of Various Conditions on Synthesis of Phosphocreatine by Muscle Tissue*

The samples were incubated with shaking at 38° in 25 cc. Erlenmeyer flasks. Each flask contained, per cc. of volume, 10 mg. of creatine, 0.125 mg. of  $Mg^{++}$ , 0.05 mg. of aspartic acid, and about 50 mg. of fresh rat muscle tissue.

Experiment No.	Other additions	Phosphate buffer		Time of incubation	Preparation of tissue	Boiled muscle juice per cc. final volume	Phosphocreatine synthesized
		M	pH				
1	0.0013 M malate	0.033	8	20	Homogenized	0	0
	0.075 M lactate						
	0.0013 M malate	0.033	8	20	"	0.3	0
	0.075 M lactate						
2*	0.0013 M malate	0.045	7.4	40	Minced	0	0
	0.075 M lactate						
	0.0013 M malate	0.045	7.4	40	"	0.3	85
	0.075 M lactate						
3	3-Phosphoglycerate†	0.033	7	10	Homogenized	0	3
	"	0.033	7	10	"	0.3	23
4	"	0.125	$Na_2HPO_4$ ‡	10	"	0	3
	"	0.125	"	10	"	0.3	44
5	"	0.033	8	10	Minced	0	52
	"	0.033	8	10	"	0.3	89

\* 150 mg. of tissue per cc.

†  $\approx$  0.4 mg. of P.

‡ Final pH = 8.7.

conditions was very erratic. Additions of boiled muscle juice made possible the transfer of phosphate from 3-phosphoglycerate to creatine by homogenates, and stimulated the transfer of minces, as shown by several experiments summarized in Table I. Increasing the pH resulted in a better synthesis of phosphocreatine, as would be expected from the results of Lehmann (10). The wide difference in the phosphorylating ability of minced and homogenized preparations, the activating effect of boiled muscle juice additions upon phosphorylation by minces, and the necessity

of boiled muscle juice additions for phosphorylation of creatine with homogenates indicated that some necessary component of the phosphorylating system was either being destroyed or diluted by the homogenizing process. Increases in the concentration of, or additions of, creatine, phosphate, adenylic acid, magnesium, or cozymase would not replace the boiled muscle juice. The effect of inorganic ions was then tried, and it was found that the addition of potassium ions resulted in a marked stimulation of phosphocreatine synthesis. With potassium present a rapid transfer of phosphate from 3-phosphoglycerate to creatine could be obtained in the absence of boiled muscle juice. This effect of potassium was manifest at pH 8.7,

TABLE II

*Effect of Potassium on Transfer of Phosphate from 3-Phosphoglycerate to Creatine*

The samples were incubated with shaking at 35° for 20 minutes. Each flask contained, per cc. of volume, 10 mg. of creatine, 0.125 mg. of  $Mg^{++}$ , 0.05 mg. of adenylic acid, 3-phosphoglycerate  $\approx$  0.4 mg. of P, and 20 mg. of homogenized, fresh rat muscle. The total volume was 2 cc.

Experiment No.	Phosphate buffer, 0.062 M	KCl added (final molarity)	NaCl added (final molarity)	Phosphocreatine synthesis  $\gamma$ P per cc.
1	$Na_2HPO_4$ *	0	0	3
	"	0.034	0	52
	"	0	0.043	0
2	pH 7.4, Na salts used	0	0	3
	" 7.4, " " "	0.034	0	11
	" 7.4, K " "	0	0	24
	" 7.4, " " "	0.034	0	21

\* Final pH = 8.7.

when maximum phosphocreatine synthesis was obtained, or at pH 7.4, and was irreplaceable by sodium, as shown in Table II.

Additions of potassium in trials in which boiled juice was present in the amounts used in the first experiments resulted consistently in a further increase in phosphocreatine synthesis, while additions of sodium were without effect. If flasks were incubated for 10 to 20 minutes with all necessary components for creatine phosphorylation except potassium, no synthesis took place, but when potassium was added a marked synthesis of phosphocreatine resulted. These results indicated that the lack of synthesis of phosphocreatine in the absence of potassium was not caused by a disappearance of some essential component.

The concentration of potassium necessary for maximum activation of the transfer of phosphate from 3-phosphoglycerate to creatine was found

to be 0.20 M, or 3 to 4 times the amount of potassium concentration in normal rat muscle, as shown in Fig. 1. The phosphorylation was markedly increased by potassium concentrations as low as 0.0125 M, and nearly completely inhibited by concentrations of 1.60 M.

In the transfer of phosphate from 3-phosphoglycerate to creatine, adenosine triphosphate is formed (17, 18). Thus the possibility that potassium might effect the transfer of phosphate from adenosine triphosphate to creatine was studied. The findings of Myers and Mangun (19) which indicated that phosphocreatine might exist in tissues as a dipotassium salt suggested that this might be the case. The results of several experiments are given in Table III. In these experiments a short incubation time was necessary because of the rapid breakdown of adenosine triphosphate by

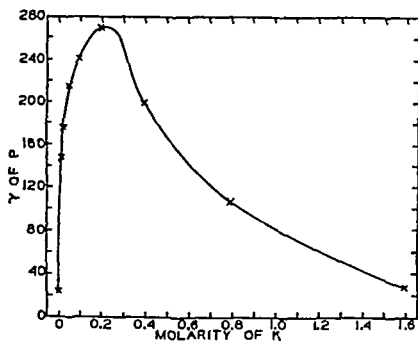


FIG. 1. The effect of potassium concentration upon the transfer of phosphate from 3-phosphoglycerate to creatine. The conditions were the same as in the experiments in Table V.

fresh muscle extracts. Sufficient adenosine triphosphate was added so that the amount of phosphocreatine formed was proportional to time for short periods when the tissue concentration was small. The transfer of phosphate from adenosine triphosphate to creatine took place equally well with or without additions of potassium. This transfer could be accomplished with tissue concentrations as low as 2 mg. per cc., while with 3-phosphoglycerate tissue concentrations of 8 to 15 mg. per cc. or above were necessary. On the basis of tissue weight the transfer of phosphate from adenosine triphosphate to creatine was 3 to 4 times more rapid than the transfer from 3-phosphoglycerate under similar conditions. Thus, these results indirectly indicated that potassium functioned in the phosphate transfer from 3-phosphoglycerate to adenylic acid.

Since 2-phosphopyruvate is first formed when creatine is phosphorylated



by 3-phosphoglycerate (10, 20, 21), the effect of potassium upon the phosphate transfer from 2-phosphopyruvate to creatine was studied. The results obtained, as given in Table IV, show that potassium is essential for the phosphate transfer, as was the case with 3-phosphoglycerate.

Calcium has been found to be inhibitory to potassium in various physiological effects (1); hence the effect of calcium on the phosphate transfer

TABLE III

*Effect of Potassium on Phosphate Transfer from Adenosine Triphosphate to Creatine*

The samples were incubated with shaking for 5 minutes at 38°. Each flask contained 10 mg. of creatine and 0.125 mg. of  $Mg^{++}$ . The total volume was 1 cc. Muscle was homogenized in the concentration of  $Na_2HPO_4$  used. The final pH was 8.7.

Experiment No.	Molarity of $Na_2HPO_4$	Adenosine triphosphate added	Tissue concentration	KCl added (final molarity)	Phosphocreatine synthesis
		mg. easily hydrolyzable P	mg. per cc.		$\gamma$ P per cc.
1	0.017	0.14	9.4	0	21
	0.017	0.14	9.4	0.025	20
2	0.10	0.14	8.3	0	35
	0.10	0.14	8.3	0.025	32
3	0.10	0.23	2.1	0	26
	0.10	0.23	2.1	0.025	27

TABLE IV

*Effect of Potassium on Phosphate Transfer from 2-Phosphopyruvate to Creatine*

The samples were incubated with shaking at 38°. Each flask contained 10 mg. of creatine, 0.125 mg. of  $Mg^{++}$ , 0.1 mg. of adenylic acid, 2-phosphopyruvate  $\approx$  0.12 mg. of P, and 12 to 13 mg. of homogenized, fresh rat muscle in 0.062 M  $Na_2HPO_4$  and 0.01 per cent NaF. The total volume was 1 cc., the final pH 8.7.

KCl added (final molarity)	Phosphocreatine synthesis	
	5 min.	10 min.
	$\gamma$ P per cc.	$\gamma$ P per cc.
0	7	12
0.20	45	90

from 3-phosphoglycerate to creatine was studied. As shown in Table V, the calcium had a definite inhibitory effect on the phosphate transfer, in concentrations much smaller than the amount of potassium present. The phosphorylation obtained in the presence of a certain calcium concentration could be increased by increasing the potassium concentration, if the original potassium concentration was less than that necessary for maximum activation (Experiment 1, Table V). However, if the optimum potassium con-

centration was present, a decrease in phosphorylation resulted from a further increase in the potassium concentration (Experiment 2, Table V). Whenever there was calcium inhibition, the synthesis of phosphocreatine was more rapid during the first than during the second 10 minute incubation period.

A limited number of experiments on the phosphate transfer from 3-phosphoglycerate to creatine have been made with acetone precipitates of fresh and dialyzed muscle extracts, prepared similarly to the procedure of Baranowski (22), and with extracts allowed to age. The acetone precipitates retained only part of the activity of the fresh extract, but did not spontaneously dephosphorylate adenosine triphosphate. The phosphate

TABLE V

*Calcium Inhibition of Phosphate Transfer from 3-Phosphoglycerate to Creatine*

The samples were incubated with shaking at 38°. Each flask contained 10 mg. of creatine, 0.125 mg. of  $Mg^{++}$ , 0.1 mg. of adenylic acid, 3-phosphoglycerate  $\approx$  0.4 mg. of P, and 12 to 13 mg. of homogenized, fresh rat muscle in 0.062 M  $Na_2HPO_4$ . The total volume was 1 cc., final pH 8.7.

Experiment No.	KCl added (final molarity)	CaCl <sub>2</sub> added (final molarity)	Phosphocreatine synthesis	
			10 min.	20 min.
			$\gamma$ P per cc.	$\gamma$ P per cc.
1	0.025	0	46	92
	0.05	0	55	104
	0.05	0.004	29	42
	0.05	0.012	2	3
	0.10	0.012	9	16
2	0.20	0	88	173
	0.20	0.002	37	50
	0.40	0.002	19	11

transfer from adenosine triphosphate to creatine by extracts prepared from acetone precipitates was not affected by potassium. However, this result by itself would be inconclusive, as potassium as well as protein is precipitated by acetone. The aging of extracts from rat muscle in several trials has resulted in a decrease in the phosphorylating activity over that obtainable with fresh extracts under optimum conditions.

## DISCUSSION

The results of these studies show that potassium has an essential rôle in muscle in the transfer of phosphate from 3-phosphoglycerate or 2-phosphopyruvate to creatine, but not from adenosine triphosphate to creatine. Hence the results of our experiments indirectly indicate that potassium is essential for the phosphate transfer from 2-phosphopyruvate

to adenylic acid. This transfer may involve two enzyme systems (22), in either or both of which potassium might function. It is possible that potassium functions in other phosphorylations, besides the phosphorylation of creatine, which involve the adenylic system.

The inhibition of the potassium stimulation of phosphorylation by calcium and the complete lack of effect of sodium are strikingly similar to results obtained in study of potassium "effects" in living tissues. Commonly used solutions such as Ringer's solution contain sufficient calcium to result in a marked inhibition. Recent results of Needham (23) and of Bailey (24) showed that calcium activated the adenosine triphosphatase activity of myosin preparations. It is possible that the inhibition by calcium may be the result of increased adenosine triphosphate breakdown. Further investigations are necessary to elucidate the mechanism of calcium inhibition.

Belitzer, in his studies on creatine phosphorylation (11), found that if the muscle mince was not washed with isotonic phosphate solution the rate of phosphorylation was greatly decreased. This effect is readily explained by a further damage to the cell and hence a further dilution of potassium. Needham and van Heyningen (9) and Lehmann (10) obtained better phosphorylation of creatine when muscle extracts were dialyzed for long periods. As they used 0.5 per cent potassium chloride solution for dialysis, the increase could be accounted for by the introduction of potassium into the extracts. These investigators also obtained better phosphorylation of creatine with old extracts. Under the conditions we used we found a decrease in activity with aging of the extracts over that obtainable with fresh extracts under optimum conditions. With optimum conditions we were able to use smaller tissue concentrations and thus introduce less material responsible for spontaneous dephosphorylation. The magnitude of the phosphorylation of creatine obtainable in our experiments, with 3-phosphoglycerate as a phosphate source, was equivalent to about 15 mg. of phosphorus as phosphocreatine per gm. of fresh tissue in 20 minutes at 38°. This is roughly 15 to 30 times as great as the synthesis obtained in 30 minutes at 20° by Needham and van Heyningen from 3-phosphoglycerate by use of dialyzed rabbit muscle extracts (9) and by Belitzer (11) who used muscle minces with lactate and malate additions. Thus under favorable conditions a high rate of phosphorylation can be obtained with fresh muscle preparations.

The necessity of potassium for phosphorylation of the adenylic system would offer a possible explanation for the observed relationships to carbohydrate metabolism (2-4, 7, 8). As potassium excess has also been shown to increase nerve respiration (5, 6), it is possible that the function of potassium in nerve may be in the phosphorylation processes. Brock and co-

workers (25) made the interesting observation that salivary gland slices did not lose their excitability after prolonged soaking in a potassium-free medium, but after a single stimulation the addition of potassium was necessary in order to renew excitability. These results could be explained on the basis that potassium was essential for the formation of phosphorylated components necessary for glandular activity, but not for the utilization of these components or the stimulation of the gland.

The lack of phosphate transfer from 3-phosphoglycerate to creatine with tissue concentrations of less than 8 to 15 mg. per cc. might possibly be due to dilution effects, discussed by Potter and Schneider (26). With tissue concentrations of 8 to 15 mg. per cc. or above, the phosphorylation obtained under optimum conditions was proportional to the tissue concentration.

#### SUMMARY

Studies on the phosphorylation of creatine by rat muscle extracts have been made and the following results obtained.

The transfer of phosphate from 3-phosphoglycerate to creatine by homogenized fresh muscle tissue with creatine, adenylic acid, magnesium, and phosphate buffer additions could not be obtained unless boiled muscle juice preparations were added. Phosphorylation by minced tissue under these conditions was erratic and was greatly stimulated by addition of boiled muscle juice. It was found possible to replace the boiled muscle juice by potassium additions. The stimulating effect of potassium was irreplaceable by sodium. Under optimum conditions a high rate of phosphorylation of creatine with fresh extracts could be obtained.

Potassium was found essential for the transfer of phosphate from either 3-phosphoglycerate or 2-phosphopyruvate to creatine, but not for the transfer from adenosine triphosphate to creatine. These results indicate indirectly that potassium acts in the phosphorylation of the adenylic system.

The transfer of phosphate from 3-phosphoglycerate to creatine in the presence of added potassium was inhibited by relatively small concentrations of calcium.

The optimum concentration of potassium for the phosphate transfer from 3-phosphoglycerate to creatine was 0.20 M. However, lower concentrations allowed a marked phosphorylation to take place. High concentrations of potassium resulted in nearly complete inhibition of the phosphate transfer.

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## LETTERS TO THE EDITORS

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### THE RÔLE OF THE AMINO ACIDS IN HUMAN NUTRITION\*

Sirs:

In previous publications from this laboratory, it has been shown that ten of the twenty-two amino acids generally recognized as components of proteins are indispensable dietary constituents for animals. Of these acids, nine are essential for growth, and even for maintenance. The tenth, arginine, is not necessary for fairly satisfactory growth, but is required for *maximum* increases in weight.

We are now engaged in determining the amino acid requirements of the human species. Two healthy young men, weighing 60 and 63.7 kilos respectively, are serving as the subjects. The make-up of the diets will be detailed at a later date. At the moment, it is sufficient to state that each diet furnishes 7.02 gm. of nitrogen daily, of which more than 95 per cent is in the form of a mixture of the ten amino acids known to be indispensable for animals. The diets differ slightly with respect to their carbohydrate content, and yield 2980 and 3190 calories per day respectively. The vitamins are supplied in daily doses of cod liver oil, and appropriate quantities of crystalline thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, nicotinamide, ascorbic acid, calcium pantothenate,  $\alpha$ -tocopherol, and 2-methyl-1,4-naphthoquinone. The unidentified factors are obtained from a concentrate of liver equivalent to 5 gm. daily of Wilson's "liver powder 1:20."

In preliminary tests, attempts were made to induce nitrogen equilibrium by diets containing 5.66 gm. of nitrogen. This level proved to be slightly too low. When the nitrogen consumption was raised to 7.02 gm. daily, equilibrium was established promptly in both subjects, and persisted throughout the fore period of 8 days. The fact that equilibrium can be maintained so readily by diets containing only ten amino acids demonstrates that *the twelve acids, previously shown to be dispensable for rats and dogs, are also dispensable for man.*

At the expiration of the fore period, valine was removed from the food, and the other amino acids were increased sufficiently to provide a constant nitrogen content. The effects of this change were profound. Immediately, each subject manifested a negative nitrogen balance. On the 4th

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day, the nitrogen output exceeded the intake by 2.19 and 2.91 gm. respectively. Valine was then returned to the food, and was followed quickly by the reestablishment of positive nitrogen balances.

After 6 days upon the complete diets, methionine was excluded from the food for a period of 6 days. Again the subjects lost more nitrogen than was ingested, although the negative balances (0.51 and 1.60, respectively) were not so large as after valine deprivation. Immediately after the administration of methionine, positive balances ensued.

The above observations demonstrate that *valine and methionine are indispensable dietary components for man*. The investigation is being continued, and the rôle of the remaining eight amino acids will be announced in the near future.

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# TRANSMETHYLATION AS A METABOLIC PROCESS IN MAN\*

Sirs:

The S-methyl group of dietary methionine has been shown to be the precursor of the methyl groups of tissue choline and creatine and of urinary creatinine in the albino rat.<sup>1</sup> In the present communication we wish to report that the process of transmethylation occurs in the human. By the isotope technique we have been able to establish that the methyl group of dietary methionine can be used by man in the synthesis of choline and creatinine.

The subject, a normal adult male (J. P. C.), ingested 6 gm. of tri-deuteriomethionine (73.3 atom per cent deuterium in the methyl group) over a period of 3 days. The test compound was taken in four 500 mg. doses at intervals during each day and 24 hour urine samples were collected. The subject consumed a diet, adequate in calories, in which the carbo-

Time	Compound isolated	Deuterium in isolated compound	Deuterium in methyl group (A)	$\frac{A}{B} \times 100$
hrs.		atom per cent	atom per cent	
0-24	Urinary creatinine	0.05 $\pm$ 0.02	0.12 $\pm$ 0.05	0.16 $\pm$ 0.06
25-48	" "	0.13 $\pm$ 0.02	0.30 $\pm$ 0.05	0.41 $\pm$ 0.06
49-72	" "	0.17 $\pm$ 0.02	0.40 $\pm$ 0.05	0.54 $\pm$ 0.06
72	Blood choline	0.89 $\pm$ 0.06	1.4 $\pm$ 0.1	1.9 $\pm$ 0.1

\* B is the deuterium content of the methyl group of ingested methionine, i.e. 73.3 atom per cent.

hydrate level was high and in which the protein, mainly of cereal origin, amounted to about 30 gm. per day.

The creatinine was isolated from each 24 hour urine sample as creatinine zinc chloride by the method of Benedict.<sup>2</sup> The purity of the salt was determined colorimetrically by the Jaffe reaction. 1.2 gm. of creatinine zinc chloride were obtained from the urine of the 1st day, 1.4 gm. from that of the 2nd day, and 0.9 gm. from that of the 3rd day.

At the close of the 72 hour experimental period 350 cc. of blood were withdrawn for the isolation of choline. The citrated blood was treated with 2 volumes of alcohol and the precipitated blood proteins were ex-

\* The authors wish to acknowledge a grant in aid by the Nutrition Foundation, Inc.

<sup>1</sup> du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., *J. Biol. Chem.*, **140**, 625 (1941).

<sup>2</sup> Benedict, S. R., *J. Biol. Chem.*, **18**, 183 (1914).



tracted three times with boiling alcohol. After the alcohol had been removed from the combined protein filtrate and extracts, the aqueous residue was treated with sufficient barium hydroxide to give a 5 per cent solution and was refluxed for 2 hours. Choline was isolated as the chloroplatinate from the hydrolysate in the usual way;<sup>1</sup> 80 mg. of choline chloroplatinate were obtained.

*Analysis*—Calculated, Pt 31.7; found, Pt 31.4

The deuterium contents of the isolated compounds are given in the accompanying table.

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